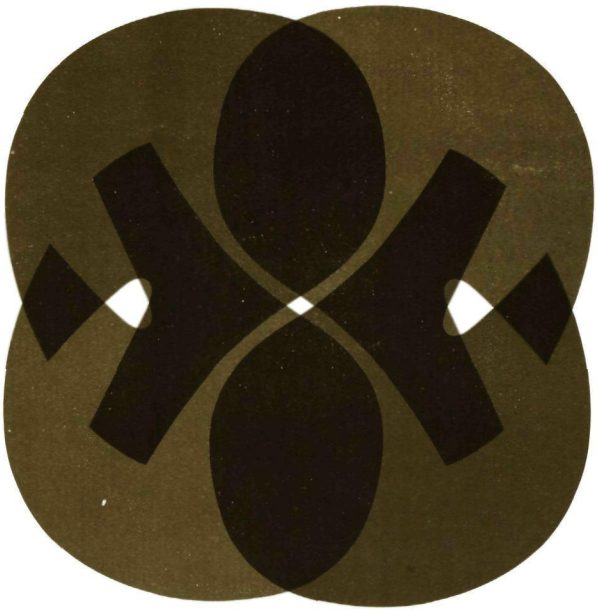

A comparative study of the normal and transformed cell surface with concanavalin A

J.G. Collard

NELLIE

A comparative study of the normal and transformed cell surface with concanavalin A

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A comparative study of the normal and transformed cell surface with concanavalin A

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VOORWOORD

Op verzoek van de faculteit Wiskunde en Natuurwetenschappen volgen hier enkele gegevens over het verloop van mijn studie

Na het eindexamen HBS-B aan het St Antonius Doctor College te Kerkrade, begon ik in september 1964 met de studie biologie aan de Katholieke Universiteit van Nijmegen In december 1967 legde ik het kandidaatsexamen af en vervolgens in februari 1971 het doctoraalexamen (cum laude) Het practisch gedeelte voor het hoofdvak werd uitgevoerd op het laboratorium voor Chemische Cytologie (Hoofd Prof Dr Ch M A Kuyper) terwijl voor de bijvakken onderzoek gedaan werd op het Zoologisch laboratorium (Hoofd Prof Dr J M Denuce) en het Botanisch laboratorium (Hoofd Prof Dr H F Linskens)

Per 1 maart 1971 trad ik als wetenschappelijk medewerker in dienst van het Nederlands Kanker Instituut (Antoni van Leeuwenhoekhuis) te Amsterdam

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Paper II Collard, J G , Temmink, J H M and Smets, L A (1975)
Cell cycle dependent agglutinability, distribution of Concanavalin A binding sites and surface morphology of normal and transformed fibroblasts In Concanavalin A, Plenum Publ Co , New York, Chapter 12, pp 221-244

Paper III Smets, L A , de Ley, L and Collard, J G (1975)
Contact-mediated changes in cytoagglutination Exptl Cell Res 95 95 102

Paper IV Collard, J G and Temmink, J H M (1974)
Binding and cytochemical detection of cell bound Concanavalin A Exptl Cell Res 86 81 86

Paper V Temmink, J H M , Collard, J G , Spits, H and Roos, E (1975)
A comparative study of four cytochemical detection methods of Concanavalin A binding sites on the cell membrane Exptl Cell Res 92 307-321

Paper VI Collard, J G and Temmink, J H M (1976)
Surface morphology and agglutinability with Concanavalin A in normal and transformed murine fibroblasts J Cell Biol 68 101-112

Paper VII Collard, J G and Temmink, J H M (1975)
Differences in density of Concanavalin A binding sites due to differences in surface morphology of suspended normal and transformed 3T3 fibro blasts J Cell Sci 19 21-32

1. INTRODUCTION

Malignancy of cells may be defined as an escape from growth, positional and immunological control. Malignant cells proliferate more or less unrestrained and are capable of migration, leading to invasive and metastatic growth. The cell surface has been found to participate, or even to play a leading role in growth regulation, tissue organization and immune control of cells. Therefore, many investigators have studied the macromolecular organization of the cell surface in order to find differences and changes that are causally related with tumorigenicity and transformation. These basic studies have mainly relied on model systems, such as normal and tumor virus-transformed normal cells cultured *in vitro*, to permit comparison of normal and malignant cells. Most of the surface changes observed after malignant transformation are schematically represented in Fig. 1. As yet, it is unclear which of the several reported surface

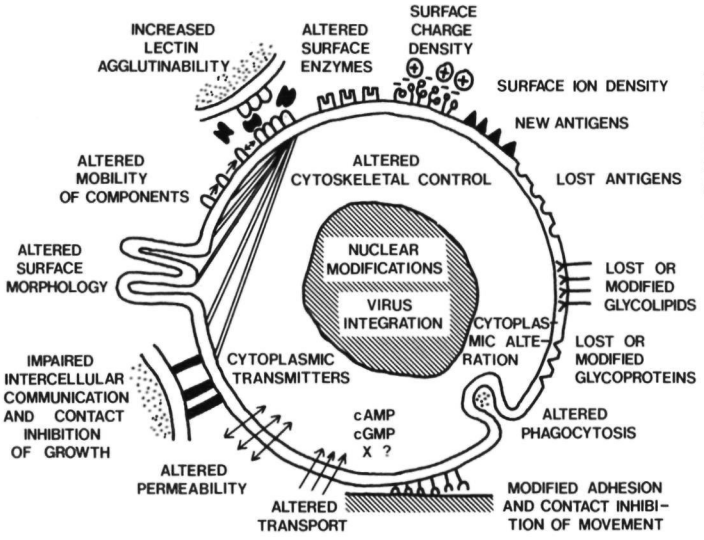

Fig. 1.

Schematic representation of some surface changes after neoplastic transformation (modified after Robbins and Nicolson, 1975).

changes are present in all transformed cell systems and which changes are only due to peculiarities of the specific model system or the specific tumor cells studied. Moreover, the relation between the various differences observed in transformed cell surfaces are often unknown and the question arises what changes are directly correlated with altered growth behaviour and what changes are a secondary result of primary changes.

In this thesis a number of papers are presented dealing with research in which the plant lectin Concanavalin A has been used to study the cell surface of normal (3T3) and SV40-transformed (SV3T3) murine fibroblasts in relation with altered growth behaviour subsequent to malignant transformation.

1.1 THE CELL SURFACE

The cell surface is loosely defined as the structure that forms the cell's outer semi-permeable barrier. It consists of the plasma membrane, the extra-cellular, cell-associated components that are bound or attached to the plasma membrane, and some intracellular components,

such as the membrane-associated cytoskeletal components (microfilaments, microtubuli) (fig. 2). The latter components have been included as part of the cell surface structure even though they may only transiently or indirectly interact with the plasma membrane.

The plasma membrane is composed of lipids, glycolipids, proteins and glycoproteins. The phospholipids and glycolipids appear to form the matrix of most plasma membranes and are arranged in a bilayer configuration as originally proposed by Gorter and Grendel (1925) and Danielli and Davson (1935). The phospholipid bilayer is not continuous, but is interrupted in certain regions by proteins and glycoproteins which can move in the plane of the plasma membrane (Singer and Nicolson, 1972).

Glycosaminoglycans (mucopeptide, mucopolysaccharide) largely make up the "glycocalyx" outside the integral zone of the plasma membrane. Glycosaminoglycans are a mixture of oligosaccharides, containing substantial amounts of hexuronic acids and hexosamines, sometimes covalently attached to small proteins.

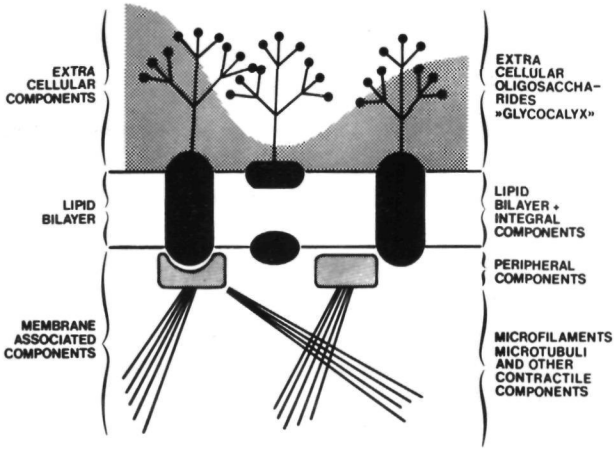

Fig. 2.

Levels of cell membrane organization.

Integral membrane components are firmly intercallated into the lipid bilayer (fig. 2). The integral membrane proteins and glycoproteins have a hydrophilic part that remains outside the lipid domain of the plasma membrane and a hydrophobic part that forms strong interactions with the membrane lipids. The glycoproteins are mainly proteins with relatively small attached oligosaccharide chains consisting largely of neutral and amino sugars including terminal sialic acid.

On the cytoplasmic side of the lipid bilayer with its integral proteins is another zone which is composed of peripheral membrane proteins (e.g. enzymes), somewhat loosely bound to the integral membrane components. Peripheral membrane proteins are not stabilized by extensive hydrophobic bonds with membrane lipids. The membrane associated zone is made up of contractile proteins like microfilaments and microtubuli that can dynamically interact with the cell membrane. The membrane associated components probably interact with the peripheral membrane proteins and have been implicated in a variety of cellular processes such as cell movement and changes in shape.

There is good evidence for the fluid nature of cell membranes and the mobility of certain membrane components (e.g. antigenic and lectin binding sites) in the plane of the plasma

membrane (fig. 3). The phospholipids are considered to diffuse laterally at a high rate in the membrane and to rapidly intermix. Certain integral proteins and glycoproteins are also able to move laterally but slower than the phospholipids, whereas other integral proteins and glycoproteins are relatively "frozen" by external restraints (cell-cell contact) or internal restraints (peripheral and membrane associated components).
For reviews on normal and transformed plasma membranes, see Emmelot, 1973; Nicolson, 1974; Robbins and Nicolson, 1975; Bretscher and Raff, 1975.

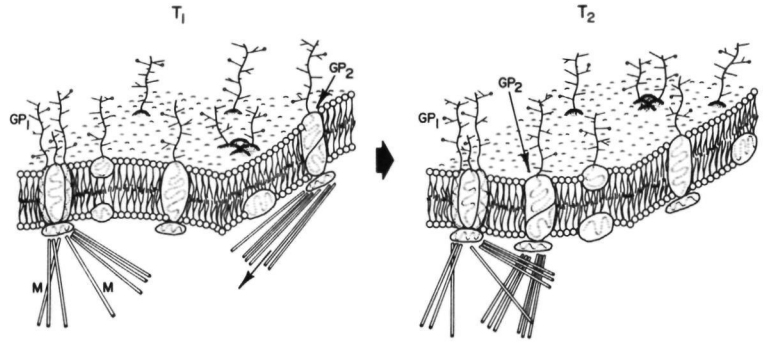

Fig. 3. Version of the fluid mosaic model of membrane structures (according to Robbins and Nicolson, 1975).
T1 and T2 represent different points in time. Certain hypothetical integral membrane glycoprotein components are free to diffuse laterally in the membrane plane formed by the fluid lipid bilayer, while others such as the integral glycoprotein complex GP1, may be impeded by a microfilament-microtubulus complex (M). Under certain conditions some integral membrane complexes (GP2) can be displaced by membrane-associated contractile components in an energy dependent process.

1.2 THE PLANT LECTIN CONCANAVALIN A.

Lectins have the property of agglutinating cells by their specific and multivalent binding to cell surface oligosaccharide determinants exposed at the cell surface. Lectins are proteins or glycoproteins that can be isolated from a wide variety of plants and animals, from legumes to horseshoe crabs. They have also been called agglutinins or hemagglutinins, but the term lectins, first used by Boyd and Shapleigh (1954) will be used here. They were originally used solely for distinguishing between blood groups, hence the name lectins (legere=select, choose). Later they were also found to distinguish between tumor cells and their untransformed parent cells.
The plant lectin Concanavalin A (Con A), prepared from the seed of the jack bean *Canavalia ensiformis*, is probably the most extensively studied plant lectin. It can be easily purified by affinity chromatography on Sephadex gels using D-glucose or sucrose in the elution buffer (Agrawal and Goldstein, 1967). Its binding specificity is directed towards a wide variety of oligosaccharides containing α-D-glucosidic or α-D-mannosidic residues (So and Goldstein, 1967 a and b). Con A is one of the few lectins that contains no covalently bound carbohydrate. Treatment of Con A with EDTA reversibly destroys its saccharide binding activity, which can be restored by addition of $MnCl_2$ and $CaCl_2$ (Yariv et al., 1968; Kalb and Levitzki, 1968; Uchida and Matsumoto, 1972). Thus divalent metal ions are required for its binding activity.

Con A is composed of identical, asymmetric subunits (Mol Wt 25,500) arranged in dimers, tetramers and higher molecular forms depending on pH and temperature (fig 4) Below pH 5 6 Con A exists in solution as a dimer with an estimated Mol Wt of 55,000, each of the two subunits contains one saccharide binding site (Kalb and Lustig, 1968, Becker et al , 1971, Hardman and Ainsworth, 1972) Above pH 5 6 Con A forms a tetramer (Mol Wt 112,000) and at pH values above pH 7 the tetrameric form further associates, forming aggregates of higher molecular weight (Agrawal and Goldstein, 1968, Kalb and Lustig, 1968) The tetrameric form of the Con A molecule is predominant at pH 7 2 at 37°C, but it dissociates into dimers as the temperature is lowered (Huet et al , 1974) A detailed description of binding specifications and physical properties of lectins is given in reviews by Sharon and Lis (1972) and Lis and Sharon (1973)

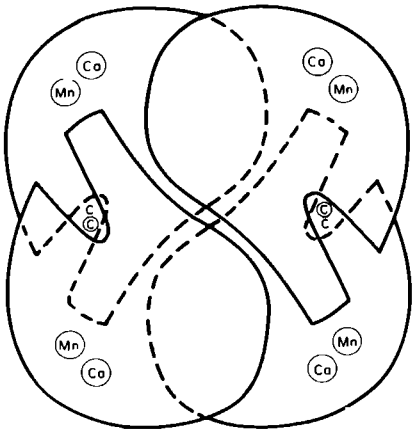

Fig 4 The tetrameric Concanavalin A molecule The proposed binding sites for carbohydrates, calcium and transition metal ions are represented by C, Ca and Mn, respectively' (From Edelman et al , 1972)

Because lectins bind specifically to certain carbohydrates, they have proved quite useful for clinical blood typing and for structural studies on blood group substances (Watkins and Morgan, 1952, Bird, 1959, Allen and Brilliantine, 1969, Boyd, 1970) Furthermore they have been applied in affinity chromatography for the specific isolation of glycoproteins, oligo- and mucopolysaccharides (Andersen, 1969, Lloyd, 1970, Dufau et al , 1972, Dorner et al , 1973) and in studies on mitogenesis in lymphocytes (Nowell, 1960, Robbins, 1964, Andersson et al , 1972, Inbar et al , 1973, Barnett et al , 1974) In addition, the reactions between lectins and their specific sites are used as antigen-antibody models (Loor, 1974, de Petris and Raff, 1974, Unanue and Karnovsky, 1974) Finally, for a number of reasons lectins like Concanavalin A can be employed as probe for the analysis of the surface architecture of normal and transformed cells Con A binds to specific sugar residues on the outside of the cell membrane and agglutinates transformed cells generally more easily than normal cells (Inbar and Sachs, 1969, Burger, 1969), increased agglutination of cells with Con A is generally correlated with higher tumorigenicity (Inbar et al , 1972, van Nest and Grimes, 1974) and loss of growth control (Burger, 1971), and cell-bound Con A can be made visible in the electron microscope by labeling with various marker molecules (Bernhard and Avrameas, 1971, Nicolson and Singer, 1971, Smith and Revel, 1972, Stobo and Rosenthal, 1972)

1 3 INTERACTIONS OF CONCANAVALIN A WITH THE CELL SURFACE OF NORMAL AND TRANSFORMED FIBROBLASTS

When normal cells *in vitro* make extensive contact with each other they stop growing This property of normal cells has been called contact or density-dependent inhibition of growth (Todaro et al , 1965, Stoker and Rubin, 1967, Dulbecco, 1970, Martz and Steinberg, 1973) It leads to the formation of monolayers of cells with a low saturation density In contrast, their transformed counterparts are capable of growing over each other and reach much higher saturation densities (Aaronson and Todaro, 1968a, Dulbecco, 1969) The term "transformed cells" is generally used for cells that have been transformed *in vitro* by a variety of agents like viruses or chemicals These cells show in tissue culture other growth characteristics than the normal cells Transformed cells have lost the capacity of contact inhibition of cell division (Aaronson and Todaro, 1968a and b, Burger, 1971) They are able to grow at serum concentrations that are too low to support the growth of untransformed cells (Holley and Kiernan, 1968, Dulbecco, 1970, Clarke et al , 1970) In addition, transformed cells are able to grow in semi-solid media, whereas normal cells can only grow when attached to a solid substrate (Macpherson and Montagnier, 1964, Stoker et al , 1968) In cases in which a direct correlation between the altered growth characteristics of transformed cells *in vitro* and the tumorigenicity of the same cells *in vivo* has been established, the terms "malignant cells" and "malignant transformation" are also legitimate (Aaronson and Todaro, 1968a, Inbar et al , 1972, van Nest and Grimes, 1974, Nicolson, 1974)

Agglutination tests show that most transformed cells agglutinate very well with low concentrations of various plant lectins such as Concanavalin A and Wheat Germ Agglutinin, whereas the normal counterparts do not (Burger, 1969, Inbar and Sachs, 1969, Ben-Bassat et al , 1971) The degree of agglutinability of cells by lectins has been found to be correlated with the saturation density of these cells (Pollack and Burger, 1969) and with the expression of the transformed state after oncogenic virus infection (Ben-Bassat et al , 1970, Sheppard et al , 1971, Lehman and Sheppard, 1972)

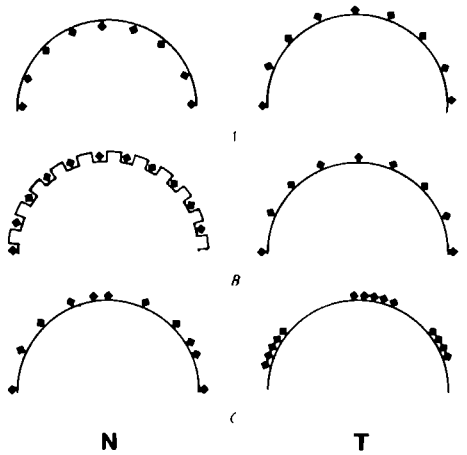

Fig 5

Three hypotheses to explain the increased lectin-mediated agglutinability after malignant transformation (from Nicolson, 1971)
A Cryptic sites theory (Burger, 1969)
B Semi-cryptic sites theory (sela et al , 1971)
C Redistribution theory (Singer and Nicolson, 1972)

Three main mechanisms (fig 5) have been proposed to explain the changes in lectin induced agglutinability that accompany malignant transformation The cryptic binding sites theory, the semi cryptic binding sites theory and the redistribution theory The theory of cryptic

binding sites has been presented by Burger (1969) and implies that lectin sites on normal cells are buried near the cell surface, but can be exposed by proteolysis or by malignant transformation (fig 5A). However, binding studies with radioactively labeled lectins did not demonstrate much difference in the number of lectin molecules bound per cell between normal, trypsinized normal and transformed cells, whereas a large difference in lectin-induced agglutinability was observed (Ozanne and Sambrook, 1971, Cline and Livingston, 1971, Inbar et al , 1972, Temmink and Collard, 1974) These data support the semi-cryptic binding sites theory presented by Sela et al. (1971) According to this theory, the lectin sites are not buried in normal cells and they can bind lectin, but cell agglutination is prevented by surface structures that can be removed by proteolysis or malignant transformation (fig 5B) More recently, the increased agglutinability of transformed cells was explained by the redistribution hypothesis presented by Singer and Nicolson (1972) Lectin binding sites are normally present in a dispersed distribution that does not favour agglutination, but after malignant transformation the lectin sites are present on the surface in a clustered distribution which favours cell agglutination (fig 5C) Later it was demonstrated that the clustered distribution of lectin sites resulted from rearrangement of these sites as induced by addition of lectin and that clustering could be prevented by prefixation or low temperature (Nicolson, 1973, Rosenblith et al , 1973)

The lectin-mediated cell agglutinability is not restricted to transformed cells only Therefore it is dangerous to make the general statement that increased cell agglutinability by plant lectins is a general property of tumor cells distinguishing them from normal cells Neverthe less, in many *in vitro* cell systems the increase in lectin agglutinability closely follows the expression of the neoplastic state, particularly in the mouse fibroblast lines, such as mouse embryo 3T3 cells compared with SV40- and polyoma-transformed 3T3 lines

1.3.1 Differences and variations in Concanavalin A-mediated agglutinability of normal and transformed fibroblasts.

(Introduction to papers I, II and III)

A mild proteolytic enzyme treatment of confluent untransformed fibroblasts *in vitro* can result in initiation of cell division and release from contact inhibition of growth (Burger, 1970, Sefton and Rubin, 1970) accompanied by an increase in agglutinability of these cells (Burger, 1973) Transformed cells are more sensitive to growth inhibition by proteolytic inhibitors than normal cells (Schnebli and Burger, 1972) Therefore, it was suggested that the changes in surface configuration of transformed cells, responsible for defective growth control and increased agglutinability, might be due to a higher proteolytic activity on the transformed cell surface (Burger, 1973) Protease inhibitors would reduce the expression of the transformed phenotype (phenotypic reversion) and thus restore a normal growth behaviour and a low lectin-mediated agglutinability (Schnebli and Burger, 1972) Our results do not support this hypothesis Protease inhibitors are not able to restore normal growth characteristics in transformed cell cultures (Collard and Smets, 1974, Paper I, Collard et al , 1975, Paper II)

The phenomenon of phenotypic reversion of transformed cells to normal growth had also been described in cells grown in media containing cyclic AMP or its derivative dibutyryl cyclic AMP (dbc-AMP) (Hsie and Puck, 1971, Johnson et al , 1971) In normal cells levels of cyclic AMP have been found to be higher than in malignant cells (Heidrick and Ryan, 1971, Sheppard, 1972) Sheppard (1971) reported that 3T3 mouse fibroblasts, transformed by polyoma virus, grow to a lower saturation density and become less agglutinable with WGA if dibutyryl cyclic AMP is added to the growth medium Therefore malignant cells were supposed to be somehow unable to maintain normal levels of cyclic AMP, but to be revertable to a normal phenotype by extracellular additions of cyclic AMP or its derivative (Sheppard, 1972) Our investigations on the effect of dbc-AMP on SV40-transformed 3T3 cells indicate that this drug, at least in our cell system did not induce true phenotypic reversion to normal growth patterns (Smets, 1972, Collard et al , 1975, Paper II)

An interesting example of phenotypic reversion of transformed cells, has been described by Burger and Noonan (1970) They treated Con A with trypsin or chymotrypsin in order to obtain "monovalent Con A" that could still bind to the cell surface but could not agglutinate through crosslinking Transformed cells, incubated with "monovalent Con A" showed phenotypic reversion to normal growth patterns, manifested by a lower saturation density and decreased agglutinability with native Con A in comparison with untreated controls They suggested that covering of agglutination sites on transformed cells with "monovalent Con A" reverses the transformed state of the cell membrane to a normal state, resulting in normal growth patterns and a decrease in saturation density of these cells However other investigators have never succeeded in repeating these results We also tried to make "monovalent Con A" by trypsination or chymotrypsination of randomly tritiated native Con A, followed by chromatography on Biogel P 100 coloms The radioactive labeling made it possible to test the binding and agglutination capacity of the different fractions obtained These results have not been published, because only the unaffected native Con A appeared active in binding and agglutination experiments The different fractions of lower molecular weight were unable to bind to the cell surface and to restore normal growth patterns in transformed cells
Recently, similar binding and agglutination experiments with the same purpose have been carried out with succinyl-Con A, prepared by extensive derivatization of Con A with succinic anhydride (Gunther et al , 1973, Trowbridge and Hilborn, 1974) Succinyl Con A has a binding specificity similar to that of native Con A but it does not readily agglutinate cells It causes a small decrease in the rate of growth and in the saturation density of both normal and transformed 3T3 cells However, in confluent transformed cells no arrest in the G_1 phase was observed (Trowbridge and Hilborn, 1974) These results make it unlikely that covering of Con A binding sites on transformed cells is sufficient to restore normal growth of these cells

Our studies on phenotypic reversion were extended with a detailed investigation of diffe rences in agglutinability of cells due to their position in the cell cycle and to intercellular contacts (Smets and de Ley, 1974, Collard et al , 1975, Paper II, Smets et al , 1975, Paper III) These studies indicate that such differences could easily interfere with the apparent occurrence of phenotypic reversion of transformed cells to normal growth patterns

1 3.2 Binding and detection of Concanavalin A on the cell surface.
(Introduction to papers IV and V)

Generally speaking, the Con A-mediated agglutinability of normal cells is low compared with that of the transformed counterparts Three main hypotheses have consecutively been advanced to explain the differences in agglutination response between normal and transformed cells (see 1 3) Because normal and transformed cells bind equal numbers of Con A molecules per cell (Ozanne and Sambrook, 1971, Cline and Livingston, 1971, Inbar et al , 1972), and Nicolson (1971) showed a cluster-like distribution of Con A binding sites on the transformed plasma membrane, the redistribution hypothesis seemed to offer the best explanation Clustering of Con A binding sites on the transformed cell surface would, according to that hypothesis, be the cause of the increased agglutination of these cells (Nicolson, 1971, 1972) However, clustering of binding sites, due to cross-linking by Con A seems to be possible in many different cell systems and may well be a universal phenomenon In addition, a distinct difference in clustering of binding sites between normal and transformed cells has not always been found Cytochemical experiments on normal and transformed hamster cells supported the alleged correlation between clustering capacity of Con A binding sites and agglutinability (Bretton et al , 1972, Martinez Palomo et al , 1972, Rowlatt et al 1973, Huet and Bernhard, 1974, Garrido et al , 1974), but results on rat cells were more ambiguous (Bretton et al , 1972, Garrido et al , 1974) and investigations on murine fibro blasts apparently contradicted the assumed correlation (Nicolson, 1971, 1972, Smith and Revel, 1972, de Petris et al , 1973)

In our investigations we studied the relationship between the total amount of cell-bound Con A as determined in binding experiments with ^{3}H-Con A and the amount and distribut ion of cell-bound Con A detected with several cytochemical markers on normal and trans formed cells (Collard and Temmink, 1974, Paper IV, Temmink et al , 1975, Paper V, Collard et al , 1975, Paper II, in part).
All experiments suggested that both normal and transformed murine fibroblasts have plasma membranes in which the binding sites can move laterally and can be induced to form clusters

1.3.3 Surface morphology and density of Concanavalin A binding sites on normal and transformed fibroblasts.
(Introduction to papers VI and VII)

In spite of the large amount of evidence for the occurrence of clustering of Con A binding sites and its occasional correlation with transformation, additional factors seem to play a role in the agglutination process Cell cycle-dependent and density dependent changes in cyto-agglutination suggested that changes in gross surface morphology of cells also play a role in the agglutination process Therefore, we investigated by scanning electron microscopy the surface morphology of both attached normal and transformed cells during the cell cycle and of cells suspended for agglutination assays (Collard and Temmink, 1976, Paper VI) In

addition, we estimated the effect of the gross surface morphology of normal and transformed 3T3 cells on the density of Con A binding sites (Collard and Temmink, 1975, Paper VII).

Our data seem to reconcile the first hypothesis (fig. 5A) of increased density of Con A binding sites on transformed cell surface with the more recent redistribution theory (fig. 5C) as a possible prerequisite for succesful agglutination of transformed 3T3 fibroblasts.

2. SUMMARY

This thesis consists of a number of papers that describe investigations on differences between the cell surfaces of normal and SV40-transformed 3T3 fibroblasts in relation with differences in growth control of these cells *in vitro* The cell surface is thought to play a leading role in growth control of cells. Nontransformed cells *in vitro* spontaneously stop dividing when they reach a certain cell density and form a monolayer of cells This phenomenon is called contact dependent inhibition of cell division In contrast, transformed cells differ from normal cells because they have lost this type of growth control Transformed cells overgrow each other and form multilayered sheets of cells *in vitro* This transformed growth behaviour has been correlated with an increased lectin-mediated agglutinability In addition, the degree of agglutinability of transformed cells has been correlated with the tumorigenicity of these cells *in vivo*

The first three papers are concerned with drug-induced changes in growth behaviour of transformed cells (phenotypic reversion to normal growth) and with modulations of the agglutinability of cells induced by Concanavalin A (Con A) In the next two papers electron cytochemical methods have been applied in attempts to visualize the Con A binding sites on the cell membrane of normal and transformed 3T3 cells and to correlate differences in distribution of these sites with differences in agglutinability The last two papers in this thesis describe the cell surface morphology of these cells and discuss its relevance to agglutination and growth

In studies on phenotypic reversion, it had been suggested that transformed cells *in vitro* could be induced to normal growth pattern by growing transformed cells in the presence of different drugs, like dibutyryl cyclic AMP or protease inhibitors Moreover, it had been reported that this phenotypic reversion was accompanied by a decrease in lectin-induced agglutinability We found that the assumed phenotypic reversion to normal growth patterns with these drugs is caused by a partial synchronization of the cells in the G_2 phase of the cell cycle The concomitant decrease in lectin-induced agglutinability was found to be a result of this accumulation of the cells in the G_2 phase of the cell cycle We showed in synchronization experiments that transformed G_2 cells have much lower agglutinability than asynchronous cells or cells synchronized in other phases of the cell cycle (Papers I and II) In addition to the finding that the agglutinability is cell cycle dependent, we also observed that the agglutinability of transformed cells is a density-dependent phenomenon Transformed cells obtained from cultures with a high cell density are more agglutinable than the same cells from a sparsely seeded culture This may indicate that the increased Con A-mediated agglutinability partly results from the transformed growth pattern (Paper III)

Furthermore, we studied the binding and distribution of Con A molecules on the normal and transformed cell surface in relation to the difference in Con A-mediated agglutinability of these cells It had been suggested that cluster-like distribution of Con A binding sites on the transformed cell surface, in contrast to the dispersed distribution on the cell surface of normal cells, causes the difference in agglutination response In our investigations, discrepancies were found between the amount of cell-bound radioactively labeled Con A and the amount of cytochemically detected Con A

It was shown that redistribution of Con A binding sites on the plasma membrane is induced

by the lectin and can be prevented by prefixation. In addition, the clustering of Con A binding sites on the plasma membrane was observed on normal as well as on transformed cells, suggesting that induced clustering of Con A binding sites may be necessary but not sufficient to explain the difference in agglutination response between normal and transformed 3T3 fibroblasts (Papers IV and V).

Our investigations and results of others indicate that many factors influence the agglutination response of cells. In this connection we also investigated the gross surface morphology of cells with the scanning electron microscope. Differences in surface morphology between normal and transformed cells *in situ* were found, especially during rounding up for mitosis. Similar differences in surface morphology were found in cells detached and suspended with EDTA for the agglutination assays. Suspended normal cells were adorned with many microvilli whereas most transformed cells were free of surface extensions (Paper VI). The difference in surface morphology between suspended normal and transformed cells affects the total cell surface area determinations of these cells and thus the actual density of the Con A binding sites per unit surface area. The number of Con A binding sites per unit surface area is about seven times larger on transformed than on normal cells. The difference in density of Con A binding sites between normal and transformed 3T3 fibroblasts could therefore be the main cause of the difference in agglutination response of these cells (Paper VII).

3. SAMENVATTING

In dit proefschrift zijn een aantal publicaties opgenomen over onderzoek naar karakteristieke veranderingen aan het celoppervlak van tumorcellen in vergelijking met normale cellen. Aan het celoppervlak wordt een belangrijke rol toegeschreven in de regulering van de groei van de cel. Bij tumorcellen is dit regelmechanisme blijkbaar verstoord, wat zich manifesteert in een min of meer onbeperkte groei. Veel onderzoek is daarom gericht op het vinden van veranderingen in het celoppervlak van tumorcellen of van kunstmatig met virus getransformeerde cellen die gepaard gaan met het veranderde groeigedrag van deze cellen *in vivo* of *in vitro*.
Als modelsysteem werd gebruik gemaakt van normale (3T3) en met SV40 (simian virus 40) getransformeerde (SV3T3) muizefibroblasten. Deze cellen worden *in vitro* gekweekt waarbij zij karakteristieke groeiverschillen vertonen. Normale cellen stoppen met groeien wanneer ze veelzijdig contact met elkaar maken en vormen een z.g. monolayer van één cellaag dik. We spreken dan van contact inhibitie van celdeling ofwel van celcultures met een lage verzadigingsdichtheid. Getransformeerde cellen daarentegen blijven wanneer ze contact met elkaar maken doorgroeien. Zij groeien over elkaar en vormen meerdere cellagen. Men spreekt dan van verlies van contact inhibitie van celdeling hetgeen leidt tot celcultures met een hoge verzadigingsdichtheid. In dit *in vitro* celsysteem is het mogelijk onderzoek te doen naar veranderingen in de structuur van de celmembraan van normale en getransformeerde cellen welke gepaard gaan met het verschil in groeigedrag als gevolg van transformatie. Hierbij werd door ons om verschillende redenen gebruik gemaakt van de plantaardige lectine Concanavaline A. Concanavaline A (Con A) wordt geïsoleerd uit zaad van *Canavalia ensiformis*. Het is een eiwitmolecuul, opgebouwd uit eenheden met twee of meer bindingsplaatsen voor specifieke suikergroepen. Con A bindt aan deze suikergroepen die op de celmembraan aanwezig zijn. Onder bepaalde omstandigheden kan door binding van Con A op verschillende cellen tegelijk een agglutinatie (celklontering) optreden. Het is gebleken dat Con A getransformeerde cellen beter doet agglutineren dan normale cellen. De verhoogde agglutinatie van getransformeerde fibroblasten d.m.v. Con A is gecorreleerd met hun tumorvormend vermogen *in vivo* en hun verlies van groeicontrole *in vitro*. Bovendien is het aan de celmembraan gebonden Con A zichtbaar te maken in de electronenmicroscoop d.m.v. verschillende marker moleculen.
In de publicaties I en II is een onderzoek beschreven over fenotypische reversie van getransformeerde cellen naar een normaal groeigedrag *in vitro*. In de literatuur werd beschreven dat getransformeerde cellen, groeiend in medium waaraan dibutyryl cyclisch AMP of protease remmers werd toegevoegd, weer een normaal groeipatroon *in vitro* gingen vertonen. Deze cellen zouden tevens een verlaagde agglutinatie laten zien. Gesuggereerd werd dat deze cellen onder invloed van deze stoffen gedwongen werden tot een normaal groeigedrag als gevolg van terugkeer naar een normale structuur van de celmembraan, gemeten aan de verlaagde agglutinatie d.m.v. Con A.
Ons onderzoek wees uit dat o.i.v. dibutyryl cyclisch AMP en protease remmers getransformeerde cellen accumuleerden in de G_2 phase van de celcyclus. Bovendien werd aangetoond dat gesynchroniseerde G_2 cellen een lagere Con A afhankelijke agglutinatie vertoonden dan cellen gesynchroniseerd in andere phasen van de celcyclus. De vermeende fenotypische reversie van getransformeerde cellen werd dus waarschijnlijk veroorzaakt door een accumula-

tie in de G_2 phase van de celcyclus tengevolge waarvan de agglutinatie d m v Con A werd verlaagd

In dit verband is in publicatie III een onderzoek beschreven over de Con A afhankelijke agglutineerbaarheid in relatie tot de dichtheid van de celculturen (= mate van contact tussen de cellen) Hierin bleek dat getransformeerde cellen gegroeid bij hoge celdichtheid beter agglutineren dan dezelfde cellen gegroeid bij een lagere celdichtheid Dit impliceert dat alleen al tengevolge van de groeiremming die optreedt tijdens fenotypische reversie van getransformeerde cellen, een verlaagde agglutinatie verwacht kan worden op grond van de contactafhankelijke agglutinatie van deze cellen.

Er zijn verschillende theorien die de verhoogde agglutinatie van getransformeerde cellen trachten te verklaren. De belangrijkste hiervan is de redistributie theorie. Deze theorie houdt in dat Con A bindingsplaatsen op de celmembraan kunnen bewegen en daardoor plaatselijk kunnen ophopen (klusteren) zodat lokaal de dichtheid van de Con A bindingsplaatsen wordt verhoogd. Klustering van bindingsplaatsen zou wel op getransformeerde maar niet op normale celmembranen optreden en zou de verhoogde agglutinatie van getransformeerde cellen verklaren

In de publikaties IV en V is een onderzoek beschreven naar de binding van radioactief Con A aan het oppervlak van normale en getransformeerde cellen Tevens zijn de gebonden Con A moleculen op het celoppervlak zichtbaar gemaakt in de electronenmicroscoop met behulp van verschillende cytochemische technieken. De resultaten wijzen uit dat redistributie van bindingsplaatsen wordt geinduceerd door toevoeging van Con A en kan worden verhinderd door de cellen vooraf te fixeren Verder bleek dat zowel op het normale als het getransformeerde celoppervlak redistributie van bindingsplaatsen optrad zodat dit niet als hoofdoorzaak gezien kan worden van het verschil in agglutineerbaarheid tussen deze cellen

Voorgaand onderzoek, betreffende celcyclus en contact afhankelijke agglutineerbaarheid van cellen, suggereerde dat de morfologie van de cel wel eens een belangrijke rol in het agglutinatie proces zou kunnen spelen Daarom bestudeerden wij de celmorfologie met behulp van de scanning electronenmicroscoop. In de publicaties VI en VII is dit onderzoek beschreven. Normale cellen zijn veel groter dan getransformeerde cellen en groeien *in vitro* met ver uitgestrekte uitlopers (leading lamellae) Gedurende het delingsproces (mitose), waarbij de cellen afronden, verschijnen talrijke microvilli op de normale cellen, terwijl dit niet of in mindere mate gebeurt op de getransformeerde cellen Ditzelfde verschijnsel doet zich ook voor wanneer de cellen kunstmatig worden afgerond voor de agglutinatietesten Blijkbaar wordt bij normale 3T3 cellen gedurende het afronden het overschot aan celmembraan opgeslagen in de vorm van microvilli. Dit verschil in celmorfologie tussen de gesuspendeerde 3T3 en SV3T3 cellen heeft belangrijke konsekwenties voor de bepaling van het totale celoppervlak van deze normale en getransformeerde cellen Het totale oppervlak van normale cellen blijkt dan veel groter te zijn dan dat van getransformeerde cellen Aangezien evenveel Con A bindingsplaatsen op getransformeerde en normale cellen worden gevonden is de dichtheid van de bindingsplaatsen op de getransformeerde cellen veel groter dan op de normale cellen Dit verschil in dichtheid van de Con A bindingsplaatsen zou een belangrijke rol kunnen spelen in het verschil in agglutineerbaarheid van de normale en getransformeerde 3T3 fibroblasten.

4 REFERENCES

Aaronson, S A and Todaro, G J (1968a) Basis for the acquisition of malignant potential by mouse cells cultivated in vitro *Science* 162 1024

Aaronson, S A and Todaro, G J (1968b) Development of 3T3-like lines from Balb/c mouse embryo cultures transformation susceptibility to SV40 *J Cell Physiol* 72 141

Agrawal, B B L and Goldstein, I J (1967) Isolation of Concanavalin A by specific adsorption on crosslinked dextran gels *Biochim Biophys Acta* 147 262

Agrawal, B B L and Goldstein, I J (1968) Protein-carbohydrate interaction VII Physical and chemical studies on Concanavalin A, the hemagglutinin of the Jack Bean *Arch Biochem Biophys* 124 218

Allen, N K and Brilliantine, L (1969) A survey of hemagglutinins in various seeds *J Immunol* 102 1295

Andersen, B R (1969) Studies on the structure of the carbohydrate moiety of rabbit γG-Globulin-I *Immunochemistry* 6 739

Andersson, J , Edelman, G M , Moller, G and Sjoberg, O (1972) Activation of B lymphocytes by locally concentrated Concanavalin A *Eur J Immunol* 2 233

Barnett, R E , Scott, R E , Furcht, L T and Kersey, J H (1974) Evidence that mitogenic lectins induce changes by lymphocyte membrane fluidity *Nature* 249 465

Becker, J W , Reeke Jr, G N and Edelman, G M (1971) Location of the saccharide binding site of Concanavalin A *J Biol Chem* 246 6123

Ben-Bassat, H , Inbar, M and Sachs, L (1970) Requirement for cell replication after SV40 infection for a structural change of the cell surface membrane *Virology* 40 854

Ben-Bassat, H , Inbar M and Sachs, L (1971) Changes in the structural organization of the surface membrane in malignant cell transformation *J Membrane Biol* 6 183

Bernhard, W and Avrameas, S (1971) Ultrastructural visualization of cellular carbohydrate components by means of Concanavalin A *Exptl Cell Res* 64 232

Bird, G W G (1959) Haemagglutinins in seeds *Brit Med Bull* 15 165

Boyd W C (1970) Lectins *Ann N Y Acad Sci* 169 168

Boyd, W C and Shapleigh, E (1954) Diagnosis of subgroups of blood groups A and AB by use of plant agglutinins (lectins) *J Lab Clin Med* 44 235

Bretscher, M S and Raff, M C (1975) Mammalian plasma membranes *Nature* 258 43

Bretton, R , Wicker, R and Bernhard W (1972) Ultrastructural localization of Concanavalin A receptors in normal and SV40 transformed hamster and rat cells *Int J Cancer* 10 397

Burger, M M (1969) A difference in the architecture of the surface membrane of normal and virally transformed cells *Proc Natl Acad Sci USA* 62 994

Burger M M (1970) Proteolytic enzyms initiating cell division and escape from contact inhibition of growth *Nature* 227 170

Burger, M M and Noonan K D (1970) Restoration of normal growth by covering of agglutinin sites on tumor cell surface *Nature* 228 512

Burger, M M (1971) The significance of surface structure changes for growth control under crowded conditions In Growth control in cell cultures (Ed G E Wolstenholme) p 45 Churchill Livingstone London

Burger, M.M. (1973). Surface changes in transformed cells detected by lectins. *Fed. Proc.* 32: 91.

Clarke, G.D., Stoker, M.G.P., Ludlow, A. and Thornton, M. (1970). Requirement of serum for DNA synthesis in BHK-21 cells: Effects of density, suspension and virus transformation. *Nature* 227: 798.

Cline, M.J. and Livingston, D.C. (1971). Binding of ^{3}H-Concanavalin A by normal and transformed cells. *Nature New Biol.* 232: 155.

Collard, J.G. and Smets, L.A. (1974). Effect of proteolytic inhibitors on growth and surface architecture of normal and transformed cells. *Exptl. Cell Res.* 86: 75.

Collard, J.G. and Temmink, J.H.M. (1974). Binding and cytochemical detection of cell-bound Concanavalin A. *Exptl. Cell Res.* 86: 81.

Collard, J.G. and Temmink, J.H.M. (1975). Differences in density of Concanavalin A binding sites due to differences in surface morphology of suspended normal and transformed 3T3 cells. *J. Cell Sci.* 19: 21.

Collard, J.G., Temmink, J.H.M. and Smets, L.A. (1975). Cell cycle dependent agglutinability, distribution of Concanavalin A binding sites and surface morphology of normal and transformed fibroblasts. In: Concanavalin A, Proc. int. Symp. Norman, Oklahoma (ed. T.K. Chowdhury and A.K. Weiss), pp. 221-244. New York: Plenum Publications.

Collard, J.G. and Temmink, J.H.M. (1976). Surface morphology and agglutinability with Concanavalin A in normal and transformed murine fibroblasts. *J. Cell Biol.* 68: 101-112.

Danielli, J.F. and Davson, H. (1935). A contribution to the theory of permeability of thin films. *J. Cell. Comp. Physiol.* 5: 495.

Dorner, F., Scriba, M. and Weil, R. (1973). Interferon: Evidence for its glycoprotein nature. *Proc. Natl. Acad. Sci. USA* 70: 1981.

Dufau, M.L., Tsuruhara, T. and Catt, K.J. (1972). Interaction of glycoprotein hormones with agarose-Concanavalin A. *Biochim. Biophys. Acta* 278: 281.

Dulbecco, R. (1969). Cell transformation by viruses. *Science* 166: 962.

Dulbecco, R. (1970). Topoinhibition and serum requirement of transformed and untransformed cells. *Nature* 227: 802.

Edelman, G.M., Cunningham, B.A., Reeke Jr, G.N., Becker, J.W., Waxdal, M.J. and Wang, J.L. (1972). The covalent and three-dimensional structure of Concanavalin A. *Proc. Natl. Acad. Sci. USA* 69: 2580.

Emmelot, P. (1973). Biochemical properties of normal and neoplastic cell surfaces: a review. *Eur. J. Immunol.* 9: 319.

Garrido, J., Burglen, M.J., Samolyk, D., Wicker, R., and Bernhard, W. (1974). Ultrastructural comparison between the distribution of Concanavalin A and wheat germ agglutinin cell surface receptors of normal and transformed hamster and rat cell lines. *Cancer Res.* 34: 230.

Gorter, E. and Grendel, F. (1925). On bimolecular layers and lipoids on the chromocytes of the blood. *J. Exptl. Med.* 41: 439.

Gunther, G.R., Wang, J.L., Yahara, I., Cunningham, B.A. and Edelman, G.M. (1973). Concanavalin A derivates with altered biological activities. *Proc. Natl. Acad. Sci. USA* 70: 1012.

Hardman, K D and Ainsworth, C F (1972) Myo-Inositol site of Concanavalin A *Nature New Biol* 237 54

Heidrick, M L and Ryan, W L (1971) Adenosine 3 ,5 cyclic monophosphate and contact inhibition *Cancer Res* 31 1313

Holley, R W and Kiernan, J A (1968) Contact inhibition of cell division in 3T3 cells *Proc Natl Acad Sci USA* 60 300

Hsie, A W and Puck, T T (1971) Morphological transformation of chinese hamster cells by dibutyryl adenosine cyclic 3′ 5 -monophosphate and testosterone *Proc Natl Acad Sci USA* 68 358

Huet, Ch and Bernhard, W (1974) Differences in the surface mobility between normal and SV40-, polyoma and adenovirus transformed hamster cells *Int J Cancer* 13 227

Huet, Ch , Lonchampt, M , Huet, M and Bernadac, A (1974) Temperature effects on the Concanavalin A molecule and on Concanavalin A binding *Biochim Biophys Acta* 365 28

Inbar, M and Sachs, L (1969) Interaction of the carbohydrate-binding protein Concana valin A with normal and transformed cells *Proc Natl Acad Sci USA* 63 1418

Inbar, M , Ben-Bassat, H and Sachs, L (1972) Membrane changes associated with malig nancy *Nature New Biol* 236 3-4 and 16

Inbar, M , Ben-Bassat, H and Sachs, L (1973) Temperature sensitive activity on the surface membrane in the activation of lymphocytes by lectins *Exptl Cell Res* 76 143

Johnson, G S , Friedman, R M and Pastan, I (1971) Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine -3 5 -cyclic monophosphate and its derivatives *Proc Natl Acad Sci USA* 68 425

Kalb, A J and Levitzki, A (1968) Metal-binding sites of Concanavalin A and their role in the binding of α methyl D-Glucopyranoside *Biochem J* 109 669

Kalb, A J and Lustig, A (1968) The molecular weight of Concanavalin A *Biochim Bio phys Acta* 168 366

Lehman, J M and Sheppard, J R (1972) Agglutinability by plant lectins increases after RNA virus transformation *Virology* 49 339

Lis, H and Sharon, N (1973) The biochemistry of plant lectins (Phytohemagglutinins) *Ann Rev Biochim* 43 541

Lloyd, K O (1970) The preparation of two insoluble forms of the phytohemagglutinin, Concanavalin A, and their interactions with polysaccharides and glycoproteins *Arch Biochem Biophys* 137 460

Loor, F (1974) Binding and redistribution of lectins on lymphocytes membrane *Eur J Immunol* 4 210

Macpherson, I and Montagnier, L (1964) Agar suspension culture for the selective assay of cells transformed by polyoma virus *Virology* 23 291

Martinez-Palomo, A , Wicker, R and Bernhard, W (1972) Ultrastructural detection of Con-canavalin A surface receptors in normal and in polyoma-transformed cells *Int J Cancer* 9 676

Martz, E and Steinberg, M S (1973) Contact inhibition of what? An analytical review *J Cell Physiol* 81 25

Nest, v G A and Grimes, W J (1974) Concanavalin A induced agglutination and tumor

igenicity in virally and spontaneously transformed cells derived from Balb/c mice *Cancer Res* 34 1408

Nicolson, G L (1971) Difference in topology of normal and tumor cell membranes shown by different surface distributions of ferritin-conjugated Concanavalin A *Nature New Biol* 233 244

Nicolson, G L. and Singer, S J. (1971) Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy Application to saccharides bound to cell membranes *Proc Natl Acad Sci USA* 68 942

Nicolson, G L (1972) Topography of membrane Concanavalin A sites modified by proteolysis *Nature New Biol* 239 193

Nicolson, G L (1973) Temperature-dependent mobility of Concanavalin A sites on tumour cell surfaces *Nature New Biol* 243 218

Nicolson, G L (1974) The interactions of lectins with animal cell surfaces *Int Rev. Cytol* 39 89

Nowell, P C (1960) Phytohemagglutinin An initiator of mitosis in cultures of normal human leukocytes *Cancer Res* 20 462

Ozanne, B and Sambrook, J (1971) Binding of radioactively labeled Concanavalin A and wheat germ agglutinin to normal and virus transformed cells *Nature New Biol* 232 156

Petris, de S , Raff, M C and Mallucci, L (1973) Ligand induced redistribution of Concanavalin A receptors on normal, trypsinized and transformed fibroblasts *Nature New Biol* 244 275

Petris, de S and Raff, M C (1974) Ultrastructural distribution of alloantigens and Concanavalin A receptors on the surface of mouse lymphocytes *Eur J Immunol* 4 130

Pollack, R E and Burger, M M (1969) Surface specific characteristics of a contact-inhibited cell line containing the SV40 viral genome *Proc Natl Acad Sci USA* 62 1074

Robbins, J H (1964) Tissue culture studies of the human lymphocyte *Science* 146 1648

Robbins, J C and Nicolson, G L (1975) In Cancer A comprehensive treatise Vol III Biology of tumors, Chapter 21 Surfaces of normal and transformed cells, Editor F F Becker, Plenum press, New York

Rosenblith, J Z , Ukena, T E , Yin, H H , Berlin, R D and Karnovsky, M J (1973) A comparative evaluation of the distribution of Concanavalin A binding sites on the surfaces of normal, virally transformed, and protease-treated fibroblasts *Proc Natl Acad Sci USA* 70 1625

Rowlatt, C , Wicker, R and Bernhard, W (1973) Ultrastructural distribution of Concanavalin A receptors on hamster embryo and adenovirus tumour cell cultures *Int J Cancer* 11 314

Schnebli, H P and Burger, M M (1972) Selective inhibition of growth of transformed cells by protease inhibitors *Proc Natl Acad Sci USA* 69 3825

Sefton, B M and Rubin, H (1970) Release from density dependent growth inhibition by proteolytic enzymes *Nature* 227 843

Sela, B-A , Lis, H , Sharon, N and Sachs, L (1971) Quantitation of n acetyl-D-galactosamine-like sites on the surface membrane of normal and transformed mammalian cells *Biochim Biophys Acta* 249 564

Sharon, N and Lis, H (1972) Lectins Cell-agglutinating and sugar-specific proteins

Science 177 949

Sheppard, J R (1971) Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3 5 cyclic monophosphate *Proc Natl Acad Sci USA* 68 1316

Sheppard, J R , Levine, A J and Burger, M M (1971) Cell-surface changes after infection with oncogenic viruses requirement for synthesis of host DNA *Science* 172 1345

Sheppard, J R (1972) Difference in the cyclic adenosine 3',5'-monophosphate levels in normal and transformed cells *Nature New Biol* 236 14

Singer, S J and Nicolson, G L (1972) The fluid mosaic model of the structure of cell membranes *Science* 175 720

Smets, L A (1972) Contact inhibition of transformed cells incompletely restored by dibutyryl cyclic AMP *Nature New Biol* 239 123

Smets, L A and de Ley, L (1974) Cell cycle dependent modulations of the surface membrane of normal and SV40 virus transformed 3T3 cells *J Cell Physiol* 84 343

Smets, L A , de Ley, L and Collard, J G (1975) Contact mediated changes in cytoagglutination *Exptl Cell Res* 95 95

Smith, S B and Revel, J P (1972) Mapping of Concanavalin A binding sites on the surface of several cell types *Dev Biol* 27 434

So, L L and Goldstein I J (1967a) Protein-carbohydrate interaction IX Application of the the quantitative hapten inhibition technique to polysaccharide-Concanavalin A interaction Some comments on the forces involved in Concanavalin A polysaccharide interaction *J Immunol* 99 158

So, L L and Goldstein, I J (1967b) Protein-carbohydrate interaction IV Application of the quantitative precipitin method to polysaccharide-Concanavalin A interaction *J Biol Chem* 242 1617

Stobo, J D and Rosenthal, A S (1972) Biologically active Concanavalin A complexes suitable for light and electron microscopy *Exptl Cell Res* 70 443

Stoker, M G P and Rubin, H (1967) Density dependent inhibition of cell growth in culture *Nature* 215 171

Stoker, M , O'Neill, C , Berryman, S and Waxman, V (1968) Anchorage and growth regulation in normal and virus transformed cells *Int J Cancer* 3 683

Temmink, J H M and Collard, J G (1974) Correlation between cytochemical detection by horse radish peroxidase and binding of Concanavalin A on normal and transformed mouse fibroblasts In Electron microscopy and cytochemistry, p 299 (Eds E Wisse, W Th Daems, I Molenaar, and P van Duyn) North Holland, Amsterdam

Temmink, J H M , Collard, J G , Spits, H and Roos, E (1975) A comparative study of four cytochemical detection methods of Concanavalin A binding sites on the cell membrane *Exptl Cell Res* 92 307

Todaro, G J , Lazar G K and Green, H (1965) The initiation of cell division in a contact-inhibited mammalian cell line *J Cell Comp Physiol* 66 325

Trowbridge, I S and Hilborn, D A (1974) Effects of succinyl Con A on the growth of normal and transformed cells *Nature* 250 304

Uchida, T and Matsumoto, T (1972) Heterogeneity of commercially available Concanavalin A with respect to carbohydrate binding ability *Biochim Biophys Acta* 257 230

Unanue E R and Karnovsky, M J (1974) Ligand induced movement of lymphocyte

membrane macromolecules *J Exptl Med* 140 1207

Watkins, W M and Morgan, W T J (1952) Neutralization of the anti-H-agglutinin in EEL serum by simple sugars *Nature* 169 825

Yariv, J , Kalb, A J and Levitzki, A (1968) The interaction of Concanavalin A with methyl α-D-glucopyranoside *Biochim Biophys Acta* 165 303

Paper I

Experimental Cell Research 86 (1974) 75–80

EFFECT OF PROTEOLYTIC INHIBITORS ON GROWTH AND SURFACE ARCHITECTURE OF NORMAL AND TRANSFORMED CELLS

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SUMMARY

Protease inhibitors were tested for their effect on the growth of normal and SV40-transformed mouse fibroblasts. The protease inhibitors TAME[1] and EWTI[1], which act competitively on proteases, reduce the growth of transformed cells more than that of untransformed parent cells. However, transformed cells grown in medium containing these drugs do not show contact inhibition of cell division or decreased agglutinability with Concanavalin A. The inhibition of growth is due to an extended duration of all phases of the cell cycle. The protease inhibitor TLCK[1], an active site titrant reacting irreversibly with trypsin, blocks transformed cells in the premitotic stage of the cell cycle. This effect does not occur in the untransformed parent cells. The decrease in agglutinability of transformed cells treated with TLCK is correlated with a partial synchronisation in the G2 stage of the cell cycle. Our results do not support the hypothesis that protease inhibitors induce transformed cells to assume a normal growth pattern and that this is accompanied by a decreased agglutinability with plant lectins.

Experimental data suggest that transformed cells differ from untransformed parent cells in the structure of their surface membrane. The generally increased agglutinability by the plant lectins Concanavalin A (ConA) and Wheat-Germ Agglutinin (WGA) [1, 2, 24] and the structural changes of the surface glycoproteins [3–5] of transformed cells correlate with the loss of growth control [6, 7] and tumorigenicity [8]. In the mitotic state, however, normal cells temporarily display a membrane structure comparable to that of transformed cells [9, 10].

Transformed cells are thought to have lost the capacity to return to the normal surface configuration after division, and according to one view this could be due to the high proteolytic activity on their surface membrane [11, 12]. A brief proteolytic treatment of untransformed fibroblasts causes increased agglutinability as well as loss of contact inhibition of cell division, suggesting that induced changes in the membrane counteract topo-inhibition of growth [7, 13].

In accord with these findings, Schnebli & Burger [11] observed that transformed cells are more sensitive to growth inhibition by proteolytic inhibitors than untransformed parent cells. They suggested that protease inhibitors induce in transformed cells a growth pattern characteristic of non-transformed cells and decreased agglutinability by the lectins WGA and ConA.

However, previous experience with induced phenotypic changes in SV-3T3 cells by dibutyryl cAMP had revealed that restora-

[1] For abbreviations used, see Materials and Methods.

tion of normal growth cannot be deduced safely from morphological criteria alone [14, 15] In order to test the hypothesis that transformed cells can be made to assume a normal growth pattern by growth in a medium containing protease inhibitors, we studied the effect of some protease inhibitors on cell cycle kinetics and surface architecture of normal and SV40-transformed 3T3 cells

MATERIALS AND METHODS

Tissue culture BALB/c mouse fibroblasts (3T3) and Simian virus 40 transformed cells (SV-3T3) came originally from Dr Todaro's laboratory Cells were grown in plastic Petri dishes (⌀ 10 cm) containing 10 ml of Dulbecco's modified Eagle medium supplemented with 10 % newborn calf serum and antibiotics in a humidified CO_2 incubator Cells were seeded at a density of 50 000 cells/ml of nutrient medium and after 24 h a protease inhibitor was added at the indicated concentration After 24 h, the culture medium was replaced by fresh medium with the same amount of inhibitor and left for another 24 h The cells were counted with a haemocytometer or with a Coulter counter and prepared for the impulse cytophotometer or they were used in the agglutination assays Control cells were grown under the same conditions except that no protease inhibitors were added to the medium

Measurement of the DNA distribution Cells were trypsinized, washed once with Tyrode salt solution and fixed as a suspension in 96 % ethanol The fixed cells were washed in Tris-HCl buffer (PH 7 3) and incubated for 20 min at 37°C with 0 1 % ribonuclease in the same buffer After washing with buffer, the cells were resuspended in 0 1 %, pepsin in 0 2 N HCl and incubated for 20 min at 37°C The cells were washed again and resuspended in Tris-HCl buffer containing 10 ppm ethidium bromide 15 min before DNA-fluorescence was measured with an impulse cytophotometer (Phywe ICP 11)

Protease inhibitors *N*-α-*p*-Tosyl-L-lysine chloromethyl ketone HCl (TLCK, Sigma) is an active site titrant that reacts irreversibly with trypsin [16] *p*-Tosyl-L-arginine methyl ester HCl (TAME, Sigma) is a substrate analogue and acts competitively on proteases and esterases [17] Egg-White Trypsin Inhibitor (Ovomucoid, B grade, Calbiochem, EWTI) forms a poorly dissociating macromolecular complex with proteases of the trypsin family [18]

Agglutination assays The cells were removed from the Petri dishes with 5×10^{-5} M EDTA in Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline (PBS), washed once with PBS containing Ca^{2+} and Mg^{2+} and suspended in PBS to a final concentration of approx 1×10^6 cells/ml For the agglutination assay 1 ml of cell suspension was transferred to a small Erlenmeyer flask on a giratory shaker at room temperature ConA (Calbiochem, A grade) was added in 0 1 ml of PBS in an amount necessary to obtain a final conc of 25 μg/ml The cell suspensions were viewed at regular intervals under a binocular microscope and the degree of agglutination was represented by a serological scale Control assays without ConA were always included

Time-lapse cinematography Cells were seeded in plastic Falcon flasks (25 cm^2) and after 24 h the culture medium was replaced by medium with or without TLCK Cell motility was filmed during the next 72 h by placing the flasks on a stage of an inverted phase microscope (Nikon) that was mounted in a polyethylene cabinet heated to 37°C with an air-incubator The cultures were photographed on Eastman Plus-X negative film with a 16 mm camera (Bolex Paillard) at 2 frames/min

RESULTS

Effects of TLCK on normal and transformed cells

TLCK at concentrations of 50 100 μg/ml of culture medium reduced the growth rate of transformed cells more than that of untransformed parent cells (table 1) To test the effect of TLCK on the cell cycle, treated

Table 1 *Effect of TLCK, TAME and EWTI on growth of normal and transformed fibroblasts during 48 h*

Cell density of 24 h of growth just prior to addition of the protease inhibitors 3T3, 13 300 cells/cm^2, SV-3T3, 13 200 cells/cm^2 Cell density after 48 h of additional growth of the control cells (without protease inhibitors, 100 %) 3T3, 54 000 cells/cm^2, SV-3T3, 61 400 cells/cm^2

Protease inhibitors	Conc (μg/ml)	No of cells in treated culture / No of cells in control culture × 100 %	
		SV-3T3	3T3
TLCK	50	50	80
TLCK	100	30	74
TAME	500	70	85
TAME	1 000	64	80
TAME	2 500	57	80
EWTI	500	86	90
EWTI	1 000	76	90
EWTI	2 500	66	85

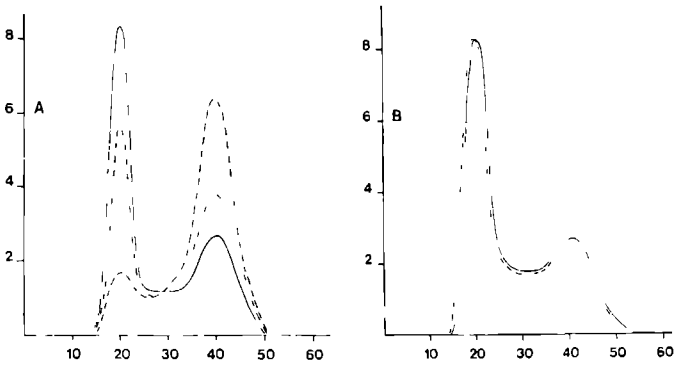


Fig. 1. Abscissa. channel no. (increasing amount of DNA/cell); *ordinate:* cells/channel ($\times 10^3$). Impulse cytophotometric distribution of cells in a culture on basis of DNA/cell content. (*A*) SV-3T3 cells; (*B*) 3T3 cells. ——, Untreated control cultures; – – –·–, cultures treated with 50 μg/ml TLCK for 48 h; – – –, cultures treated with 100 μg/ml TLCK for 48 h.

cultures were prepared for measurement of the DNA distribution per cell. TLCK arrested SV-3T3 fibroblasts in the premitotic state of the cell cycle, whereas 3T3 parent cells did not show this effect (fig. 1 *A*, *B*). Moreover, polyoma-transformed BHK cells (PY-BHK) and spontaneously transformed 3T3 cells (3T3-f) also showed accumulation in the premitotic state by TLCK (100–200 μg/ml) in contrast to the non-transformed parent cells (not shown).

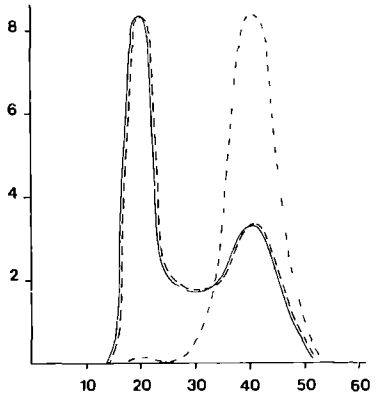
Whether TLCK acted directly on the cells or by modifying serum factors in the growth medium was investigated next. Serum was incubated with TLCK (200 μg/ml) for 2 h at 37°C. The inhibitor was removed by dialysis against PBS for 72 h and the dialysed serum was used to prepare complete growth medium. No changes in growth of SV-3T3 and 3T3 cells were observed in this medium as compared with growth in a culture medium supplemented with untreated dialysed serum. Also, no changes in the DNA distribution per cell were measured in transformed cells grown in medium supplemented with TLCK-treated serum. Apparently TLCK did not affect the growth by modifying serum factors in the culture medium.

Cinematographic studies showed a decrease of motility for 3T3 and SV-3T3 cells grown in medium containing TLCK (100 μg/ml). Transformed cells rounded up in preparation of division but returned subsequently to the premitotic state by stretching (compare fig. 1 *A*). Untransformed cells could still complete the mitotic division process but the duration of mitosis had increased 5–20 times.

In conclusion: TLCK does not induce transformed cells to grow like normal cells. The observed reduction in growth rate in SV-3T3 cells is caused by accumulation in the premitotic state of the cell cycle. The small reduction in growth rate in untransformed 3T3 cells is probably due to a proportional extension of all phases of the cell cycle, because no accumulation in a particular phase could be observed.

Effects of TAME and EWTI on normal and transformed cells

TAME and EWTI are not active site titrants like TLCK but their inhibitory effect is reversible. Nevertheless TAME and EWTI also reduced the growth rate of transformed cells more than that of normal cells when added to the culture medium in concentrations up to 2 500 μg/ml (table 1). However, the distribution of cellular DNA contents indicated that transformed and untransformed cells grown with TAME or EWTI (1 000 μg/ml) did not accumulate in any particular part of the cell cycle in contrast to transformed cells grown in TLCK.



Figs 2–3. Abscissa: channel no. (increasing amount of DNA/cell); *ordinate:* cells/channel (× 10^3). Impulse cytophotometric distribution of SV-3T3 cells in a culture on basis of DNA/cell content. —, Untreated control cultures; ----, cultures grown for 48 h in medium containing 500 μg/ml TAME; -·- - -, cultures grown for 48 h in medium containing 500 μg/ml TAME and irradiated with 1 800 R after 24 h in TAME-containing medium.

However the possibility still existed that the transformed cells were contact inhibited in G1 but went undetected because of the size of the G1 fraction. In order to exclude this possibility subconfluent transformed cells were grown during 24 h in medium containing TAME or EWTI and subsequently irradiated with 1 800 R of X-rays. After another 24 h of growth in this medium all cells had accumulated in the G2 stage of the cell cycle due to irradiation induced mitotic delay (fig. 2). No cells had been arrested in the G1 stage as would have been expected if 'contact-mediated' growth had been induced by the inhibitor. Irradiation of confluent 3T3 cells alone caused no release of the contact inhibition of cell division (fig. 3). Therefore it is concluded that the reduction of growth rate by TAME or EWTI of transformed cells is due to a prolongation of the various phases of the cell cycle and not to induced contact mediated growth control.

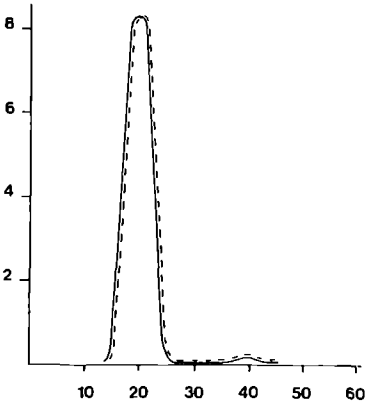


Fig. 3. Impulse cytophotometric distribution of confluent 3T3 cells with and without irradiation on basis of DNA/cell content. -.-.-.-, Culture grown 24 h after irradiation with 1 800 R; ——, control culture without irradiation.

Table 2. *Changes in agglutinability of SV-3T3 cells after treatment with different protease inhibitors for 48 h or with 1 800 R of X-rays 24 h prior to testing*

Treatment	% cells in G2	Agglutination after 20 min
Control	30	+ + + +
Tame, 500 μg/ml	31	+ + + +
EWTI, 500 μg/ml	30	+ + + +
TLCK, 100 μg/ml	62	+ + +
X-rays	85	+ +

Effects of the protease inhibitors on agglutinability

SV-3T3 cells were grown with the protease inhibitors to test the effect of the drugs on agglutinability with ConA. Other cells were treated with X-rays for the same purpose. After suspending the cells, part of the suspension was used to measure the distribution of the cellular DNA by impulse cytophotometry and part was used for the agglutination test. From the DNA distributions the percentage of G2 cells was calculated (table 2).

TLCK and X-ray treatment caused the cells to accumulate in G2 phase, whereas

TAME and EWTI did not In addition TLCK and X-ray treatment reduced the agglutinability of SV-3T3 cells, contrary to TAME and EWTI Therefore TLCK probably does not reduce the agglutinability of SV-3T3 cells directly but only indirectly by its effect on the cell cycle

DISCUSSION

In the present investigation, cell cycle parameters were studied on normal and transformed fibroblasts grown in the presence of protease inhibitors These inhibitors have been reported to affect preferentially transformed fibroblasts They reduce the rate of proliferation, change the morphology towards a more flattened appearance resembling that of untransformed cells and in the case of TLCK decrease the agglutinability with WGA and ConA [11]

These findings have been interpreted as indicating that protease inhibitors restore in transformed cells the normal phenotype with regard to growth pattern and agglutination with plant lectins This conclusion is complementary to earlier findings as to the release from contact inhibition of cell division and the increase in agglutinability of normal cells treated with proteolytic enzymes [6, 7, 13]

Our results do not support this concept of reversion to normal growth behaviour by protease inhibitors Such reversion would imply that the treated cells accumulate in the G1 stage of the cell cycle due to restoration of contact inhibition of growth However, we found that SV-3T3 cells accumulated in G2 phase in the presence of TLCK (fig 1*A*) or were equally distributed over all phases of the cell cycle with TAME or EWTI In fact, all cells grown in the presence of TAME or EWTI were able to accumulate at a radiation-induced block in G2, indicating that no cells had been trapped in G1 by contact inhibition (fig 2)

TLCK caused transformed cells to accumulate in G2, whereas TAME and EWTI did not. Apparently the effect of TLCK on the cells is different from that of other inhibitors This could be due to the fact that TLCK is an alkylating agent and many alkylating agents are known to exhibit radiomimetic properties [19] Cinematographic studies clearly showed the effect of TLCK on transformed cells Treated cells made several attempts to round up for division but than stretched again on the substrate without cell division The motility of the cells was strongly reduced

Table 2 demonstrates that the decrease in agglutinability correlates with a shift in the population to increased numbers of cells in G2 phase This effect is obtained only with TLCK and not with TAME or EWTI Consequently, G2 accumulation by TLCK is most likely not due to inhibition of proteolytic modification of the cell periphery but to the alkylating and radiomimetic properties of TLCK

The preferential inhibition of transformed cell growth by inhibitors of proteolytic enzymes (table 1) confirms the results of Schnebli & Burger [11] According to our results, this effect is due to inhibition of the growth rate of transformed cells throughout the cell cycle rather than to restoration of growth control Consequently, proteolytic activity at or near the cell membrane seems essential for optimal growth of transformed cells [24] This might be correlated with the high content of catabolic enzymes observed in the interstitial fluid of tumour cells [26]

Normal cells are highly agglutinable during mitosis but return to low agglutinability very soon after cell division [9] Transformed cells are supposed to display permanently the mitotic surface configuration However,

there are indications that transformed cells in G2 phase have the same low agglutinability as normal cells in interphase Incubation of cells with dibutyryl cAMP causes accumulation in the premitotic phase in SV-3T3 cells [14, 15] and a decrease in agglutinability with plant lectins in other transformed cell lines [20, 21] These findings agree with our results obtained with the protease inhibitor TLCK (table 2) A cell cycle-dependent change in agglutinability has been found in long-term cultures of human lymphocytes transformed by Epstein-Barr virus [22] In this cell system, populations of G2 cells have a lower agglutinability then asynchronous or G1 populations of these cells

It is not yet clear whether the decrease in agglutinability of transformed cells by TLCK is causally related to G2 accumulation There are several possible explanations Perhaps G2 cells are less agglutinable by an increase in cell size and therefore a decrease in Con A-binding sites per unit of cell surface Another possible explanation is that drugs such as TLCK or dibutyryl cAMP decrease the membrane mobility which causes G2 accumulation in some cell lines and independently a decrease in agglutinability by inhibition of clustering of the Con A-binding sites [23, 24] We observed a strong inhibition of cell mobility caused by TLCK similar to the effect of dibutyryl cAMP [14, 25] Cell cycle-dependent agglutinability of normal and transformed cells is presently studied in order to discriminate between these alternatives

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REFERENCES

1 Burger, M M & Goldberg, A R, Proc natl acad sci US 57 (1967) 359
2 Inbar, M & Sachs, L, Nature 223 (1969) 710
3 Buck, C A, Glick, M C & Warren, L, Biochemistry 9 (1970) 4567
4 — Science 172 (1971) 169
5 van Beek, W P, Smets, L A & Emmelot, P, Cancer res 33 (1973) 2913
6 Burger, M M, Nature 227 (1970) 170
7 — Growth control in cell cultures (ed G E W Wolstenholme & J Knight) Churchill-Livingstone, London (1971)
8 Inbar, M, Ben Bassat, H & Sachs, L, Nature new biol 236 (1972) 3
9 Fox, T O, Sheppard, J R & Burger, M M, Proc natl acad sci US 68 (1971) 244
10 Glick, M C & Buck, C A, Biochemistry 12 (1973) 85
11 Schnebli, H P & Burger, M M, Proc natl acad sci US 69 (1972) 3825
12 Poste, G, Exptl cell res 73 (1972) 319
13 Burger, M M, Proc natl acad sci US 62 (1969) 994
14 Smets, L A Nature new biol 239 (1972) 123
15 Paul, D, Nature new biol 240 (1972) 179
16 Shaw, W, Mares-Guia, M & Cohen, W, Biochemistry 4 (1965) 2219
17 Kassel, B & Laskowski, M, J biol chem 219 (1956) 203
18 Fraenkel-Conrat, H, Bean, R S & Lineweaver, H, J biol chem 177 (1949) 385
19 Bacq, Z M & Alexander, P, Fundamentals of radiobiology, 2nd edn, p 217 Pergamon Press, London (1961)
20 Sheppard, J R, Proc natl acad sci US 68 (1971) 1316
21 Hsie, A W, Jones, C & Puck, T T, Proc natl acad sci US 68 (1971) 1648
22 Smets, L A, Nature new biol 245 (1973) 113
23 Nicolson, G L, Nature new biol 233 (1971) 244
24 Burger, M M, Fed proc 32 (1973) 91
25 Johnson, G S, Morgan, W D & Pastan, I, Nature 235 (1972) 54
26 Sylven, B & Bois-Svensson, S, Cancer res 25 (1965) 468

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CELL CYCLE DEPENDENT AGGLUTINABILITY, DISTRIBUTION OF CONCANAVALIN A BINDING SITES AND SURFACE MORPHOLOGY OF NORMAL AND TRANSFORMED FIBROBLASTS.*

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ABSTRACT

In studies on phenotypic reversion of transformed cells to normal growth patterns, we investigated the effect of dibutyryl cyclic AMP (dbc-AMP) and a protease inhibitor (TLCK) on growth of SV40-transformed mouse fibroblasts (3T3). The results did not support the hypothesis that transformed cells grown with dbc-AMP or TLCK are induced to contact-mediated growth control. The growth rate of SV-3T3 cells grown with the drugs was strongly reduced, due to accumulation of the cells in the G_2 phase of the cell cycle. In addition, decreased agglutinability with concanavalin A (Con A) of those SV-3T3 cells was not caused by a direct effect of the drugs on the cell surface, but by partial synchronization of the cells in the G_2 phase of the cycle. In synchronized cultures agglutinability of transformed cells reached a minimum in G_2 and was maximal in mitosis and G_1. Normal cells agglutinated only in mitosis. This suggested that agglutinability of cells is somehow cell cycle dependent.

Cytochemical investigations on normal and transformed 3T3 cells had shown that Con A-induced redistribution of binding sites on the surface of these cells is not correlated with agglutinability. The present work on replicas confirmed this, but indicated also that normal 3T3 cells have more extended lamellipodia with less Con A binding sites than SV-3T3 cells. Preliminary scanning electron

*The facilities for the scanning electron microscopy was available through the Department of Electron Microscopy of the University of Amsterdam.

microscope data showed cell cycle dependent changes in 3T3 cells and also showed that confluent 3T3 and SV-3T3 cells suspended for agglutination tests had a different surface morphology. These results may represent additional factors important for differences in cell agglutinability by Con A.

I. INTRODUCTION

The plasma membrane is thought to play an important role in growth regulation of cells. The plant lectin concanavalin A (Con A) is known to bind to the cell membrane and to agglutinate transformed cells more easily than normal cells (Inbar and Sachs, 1969). Increased agglutination with Con A correlates generally with tumorgenicity (Inbar *et al.*, 1972) and loss of growth control (Burger, 1970; Burger, 1971). Con A binds to specific sugar residues on the outside of the cell membrane and can be made visible in the electron microscope by labeling with various marker molecules (Bernhard and Avrameas, 1971; Nicolson and Singer, 1971; Smith and Revel, 1972; Stobo and Rosenthal, 1972). Therefore we used Con A in previous studies on differences in the structure of the cell membrane between normal and SV40-transformed mouse fibroblasts in relation to differences in growth control *in vitro* (Collard and Temmink, 1974; Temmink and Collard, 1974; Temmink *et al.*, 1975). These studies and similar investigations by others (Nicolson, 1971; Comoglio and Guglielmone, 1972, Bretton *et al.*, 1972; Martinez-Palomo *et al.*, 1972, de Petris *et al.*, 1973, Garrido *et al.*, 1974; Huet *et al.*, 1974; Rowlatt *et al.*, 1974) led to a number of apparently conflicting results regarding the correlation between changes in growth control and agglutinability on the one hand, and changes in amount or distribution of Con A binding sites on the cell membrane on the other hand.

In the literature it has been suggested that dibutyryl cyclic AMP (dbc-AMP) (Hsie and Puck, 1971; Johnson *et al.*, 1971) and protease inhibitors (Schnebli and Burger, 1972) induce normal growth patterns in transformed cells and that transformed cells grown with these reagents have a low agglutination with plant lectins (Sheppard, 1971; Schnebli and Burger, 1972). For that reason we studied phenotypic reversion of transformed cells to normal growth patterns, as induced by dbc-AMP and protease inhibitors. We were able to confirm that the growth rate of SV40-transformed 3T3 cells (mouse fibroblast) is strongly reduced by dbc-AMP (Smets, 1972) or protease inhibitors (Collard and Smets, 1974). Our results indicate, however, that the reduction in growth rate is correlated with an accumulation of the cells in the G_2 phase of the cell cycle and not in G_1, as would have been expected if contact mediated growth had been induced (Smets, 1972; Collard and Smets, 1974).

This suggested that the position of cells in the cell cycle may

be an important factor in agglutination. This had been confirmed by our observations on cell cycle dependent changes in agglutinability of Ebstein-Barr virus transformed lymphocytes (Smets, 1973). Therefore we tried to find similar changes in agglutination during the cell cycle in synchronized cultures of normal and SV40 virus-transformed fibroblasts. The results of this study and other investigations (Rubin and Everhart, 1973; Porter *et al.*, 1973a,b) demonstrate that the overall surface morphology of some cells changes during the cell cycle. This supports the hypothesis that differences in agglutinability by Con A between normal and transformed cells do not only result from assumed differences in mobility of Con A binding sites, but also from differences in the gross surface morphology between these cells. Some scanning and transmission electron microscope experiments described in this report give additional circumstantial evidence for this notion and will be discussed in relation to the existing theories on cell agglutination by Con A and growth control *in vitro*.

II. MATERIALS AND METHODS

A. Tissue Culture

Mouse 3T3 fibroblasts and SV40 virus-transformed fibroblasts (SV-3T3) were obtained commercially from Flow Laboratories, U. S. A. Cells were grown in plastic Petri dishes (10 cm dia.) containing 10 ml of Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and antibiotics in a humidified CO_2 incubator at 37°C. In the experiments, cells were seeded at a density of 5 x 10^4 cells/ml of nutrient medium. After 24 hours of growth the culture medium was replaced by fresh medium with the different drugs as indicated in the experiments. Control cells were grown under the same conditions except that no drugs were added to the medium. For the electron microscopic studies, cells were grown on cover slips in plastic Petri dishes (5 cm dia.), filled with 5 ml of culture medium.

B. Agglutination Assays

Cells were removed from the Petri dishes with 5 x 10^{-5} M EDTA in Ca^{++} and Mg^{++} free phosphate buffered saline (PBS), washed once with PBS containing Ca^{++} and Mg^{++} and suspended in PBS to a final concentration of approx. 1 x 10^6 cells/ml. The agglutination assays were performed at room temperature with 1 ml of cell suspension in a small Erlenmeyer flask on a gyratory shaker. Con A (Calbiochem, A grade) was added in 0.1 ml of PBS to an amount necessary to obtain a final concentration of 25 µg/ml. Maximal agglutination in less than 20 min was scored as (+++++) without further differentiation and incomplete agglutination in 20 min was recorded on the usual scale (0 - ++++). In all experiments cell suspensions without Con A were used as the controls.

C. Measurement of the DNA Distribution

Cells were suspended with EDTA or trypsin, washed once in tyrode salt solution and fixed in suspension by adding an excess of 96% ethanol. The fixed cells were washed in Tris-HCl buffer (pH 7.3) and incubated for 20 min at 37°C with 0.1% ribonuclease in the same buffer. After washing with buffer the cells were resuspended in 0.1% crude pepsin in 0.2 N HCl and incubated for 20 min at 37°C. The cells were washed again and resuspended in Tris-HCl buffer containing 10 ppm ethidium bromide 15 min before DNA fluorescence was measured with a Phywe ICP 11 impulse cytophotometer. With this apparatus, the DNA content of at least 20,000 cells was measured, stored proportionally in a 120 channel analyzer, and plotted on a frequency distribution curve.

D. Description of Different Drugs

Dibutyryl adenosine 3':5'-cyclic monophosphate (dbc-AMP) was obtained from Calbiochem and theophylline from Merck. The protease inhibitor N-α-p-Tosyl-l-Lysine chloromethyl ketone HCl (TLCK, Sigma) is an active site titrant that reacts irreversibly with trypsin. *p*-tosyl-l-arginine methyl ester HCl (TAME, Sigma) is a substrate analogue and acts competitively on proteases and esterases. Egg-White Trypsin inhibitor (EWTI, Ovomucoid, B grade, Calbiochem) forms a poorly dissociating macromolecular complex with the proteases of the trypsin family (Schnebli and Burger, 1972).

E. Synchronization Procedures

Cells in mitosis were isolated by the shake-off method (Terasima and Tolmach, 1963) and early G_1 cells were obtained following plating of the mitotic cells for 1-2 hours in culture dishes. Populations of cells at the G_1/S boundary were obtained by synchronization in excess thymidine (5 mM) or fluoro-uridine (FUdr) (20 μg/ml). For this purpose, SV-3T3 cells from stationary cultures were inoculated at a density of 1.5×10^5 cells per cm^2 and grown for 18 hours in medium containing the inhibitor. In the case of 3T3 cells, the cultures were inoculated with cells from confluent monolayers (saturation density: $5 \times 10^4/cm^2$) at half saturation density and the serum concentration was raised to 20%. Early and late S cells were obtained from cultures synchronized with excess thymidine, by washing the cells twice with prewarmed regular medium and growing them in this medium for 3 and 7 hours respectively.

To prepare populations of pure G_2 cells, cultures were initiated as described for synchronization in S phase, with omission of the inhibitors. The cultures were irradiated with 1800 Roentgen of X-rays some 5 hours after inoculation and collected 24 hours thereafter. At that time, more than 90% of the cells had accumulated at

the radiation-induced block in G_2. Aliquots from all preparations of synchronized cells were subjected to cytophotometry to calculate the average position of the cells in the cycle. Details of histogram analysis have been published elsewhere (Smets, 1973).

F. Transmission Electron Microscopy of Replicas

Cells grown on cover slips were rinsed, then treated *in situ* with Con A (50 µg/ml) and hemocyanin (HC) (500 µg/ml) for 15 min at 25°C, washed, and fixed for 30 min in 2.5% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.2). After dehydration in ethanol and amyl acetate the material was dried in a blast of warm air from a hair dryer. The compressing effect on the cells of this drying method is advantageous for transmission electron microscopy, where little depth of field is available. The preparation of the replicas was done according to the method described by Smith and Revel (1972).

G. Scanning Electron Microscopy

Cells on cover slips or suspended cells attached to a confluent monolayer on cover slips were washed and fixed for 30 min in 2.5% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.2). The suspended cells were prepared with 5 x 10^{-5} M EDTA in Ca^{++} and Mg^{++} free PBS, washed twice with PBS containing Ca^{++} and Mg^{++}, and prefixed after 15 min in 2.5% glutaraldehyde. After fixation for 1 h, the suspended cells were washed three times in PBS and layered on cover slips with a monolayer of confluent 3T3 cells. After 4 h at 37°C the cover slips with attached cells were fixed as described. After dehydration in ethanol and amyl acetate the cells were dried by the critical point method (Anderson, 1951) with CO_2. The dried cells on glass were mounted on stubs and covered with gold in a Balzer freeze etching apparatus. The material was studied in a Cambridge Stereoscan.

III. RESULTS

A. Effect of dbc-AMP and Protease Inhibitors on Normal and Transformed Cells

Previous experiments on phenotypic changes in SV-3T3 cells induced by dbc-AMP and protease inhibitors had revealed that restoration of normal growth patterns cannot be deduced safely from the phenotypic appearance in a light microscope only (Paul, 1972; Smets, 1972). Therefore we studied the effect of dbc-AMP and protease inhibitors on cell cycle kinetics and agglutinability of normal and SV 40-transformed 3T3 cells.

For that purpose, cells were grown during 48 hours with different drugs and subsequently prepared for cytophotometry and ag-

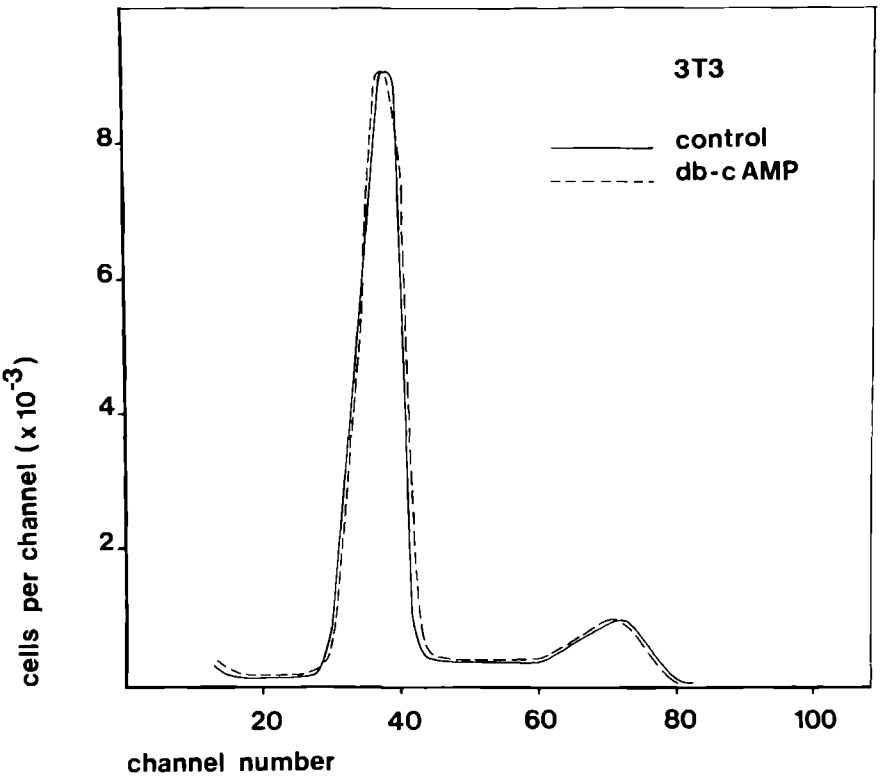

Fig. 1. Impulse cytophotometric distribution of 3T3 cells, grown during 48 hours with and without db-c AMP, on basis of DNA/cell content.

glutination tests. Fig. 1 and 2 represent the impulse cytophotometric diagrams of 3T3 and SV-3T3 cells grown during 48 hours in 10^{-3} M dbc-AMP and 10^{-3} M theophylline. Fig. 3 and 4 show the corresponding diagrams for cells grown during 48 hours with the protease inhibitor TLCK. It is apparent that transformed cells grown with these drugs accumulate in the G_2 phase of the cell cycle, whereas normal cells do not. Thus the reduced growth of transformed cells with these drugs is correlated with preferential retardation of the cells in the G_2 phase of the cell cycle and not in G_1 as would be expected if contact mediated growth had been induced.

When transformed cells, grown for 48 hours in dbc-AMP or in different protease inhibitors, were tested for agglutination, we found a correlation between amount of G_2 cells per culture and decrease in agglutinability (Table I). TLCK, dbc-AMP and X-ray

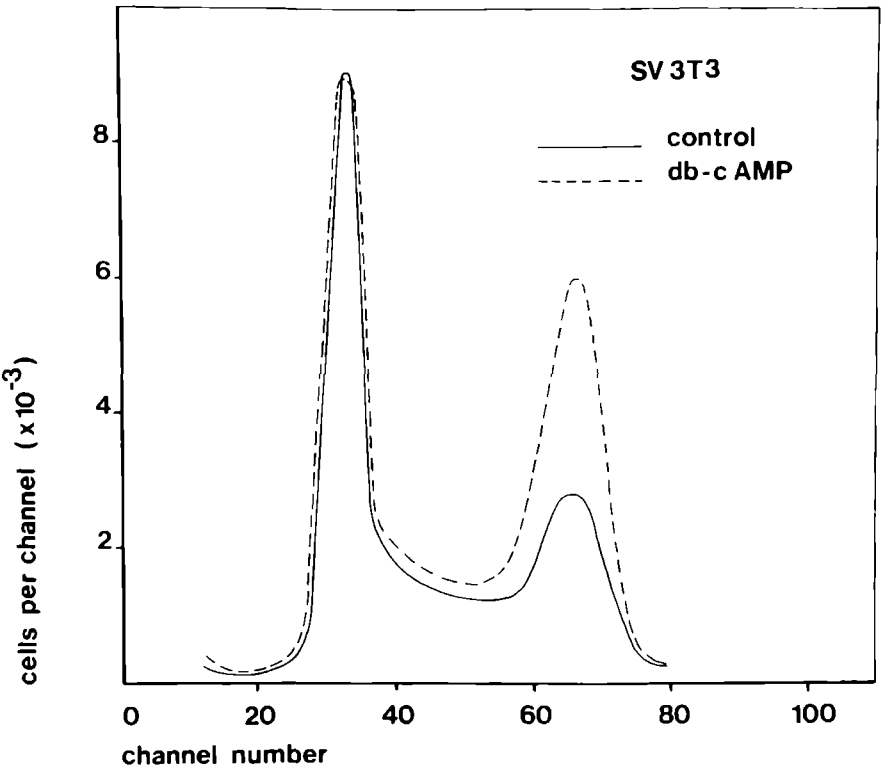

Fig. 2. Impulse cytophotometric distribution of SV-3T3 cells, grown during 48 hours with and without db-c AMP, on basis of DNA/cell content.

TABLE I

CHANGES IN AGGLUTINABILITY OF SV-3T3 CELLS AFTER TREATMENT WITH DIFFERENT PROTEASE INHIBITORS AND dbc-AMP FOR 48 HOURS OR WITH 1800 R OF X-RAYS 24 HOURS PRIOR TO TESTING.

Treatment	% cells in G_2	Agglutination after 20 min.
Control	30	+ + + +
TAME, 500 µg/ml.	28	+ + + +
EWTI, 500 µg/ml.	30	+ + + +
TLCK, 100 µg/ml.	63	+ + +
dbc-AMP 10^{-3} M.	46	+ +
X-rays	85	+ +

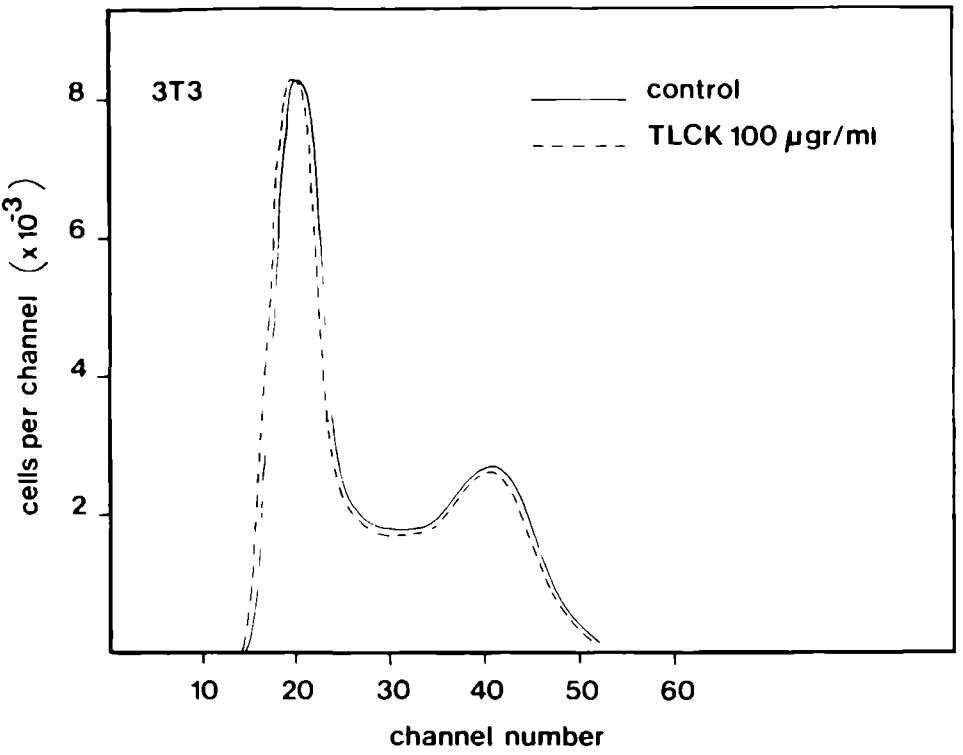

Fig. 3. Impulse cytophotometric distribution of 3T3 cells, grown during 48 hours with and without TLCK, on basis of DNA/cell content.

TABLE II

AGGLUTINATION WITH CON A OF 3T3 AND SV-3T3 CELLS IN VARIOUS PHASES OF THE CELL CYCLE.

Cycle phase	Agglutination after 20 mins at 25 µg/ml. 3T3 cells	SV-3T3 cells
Mitosis	+ + + + +	+ + + + +
Early G_1	+	+ + + +
G_1/S	0	+ + + +
Early S	0	+ + +
Late S	0	+(+)
G_2	0	+(+)
Asynchronous cells	0	+ + + +

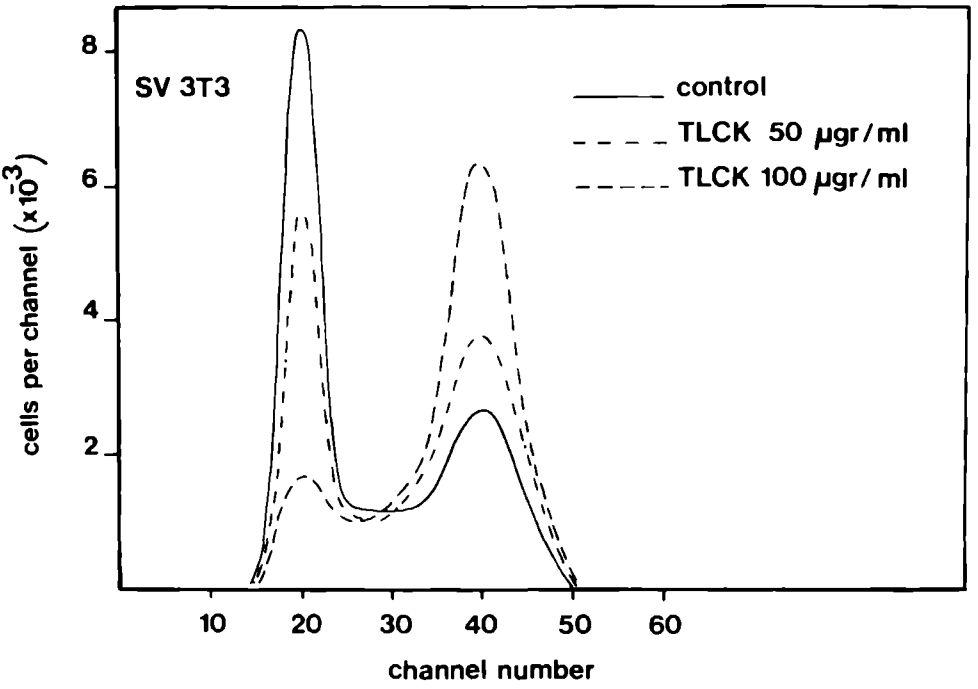

Fig. 4. Impulse cytophotometric distribution of SV-3T3 cells, grown during 48 hours with and without TLCK, on basis of DNA/cell content.

treatment caused the cells to accumulate in the G_2 phase of the cell cycle, whereas TAME and EWTI did not. Thus, the agglutination is reduced only by those drugs that induce an accumulation in the G_2 phase, and we suppose that this partial synchronization caused the decrease in agglutination with Con A.

B. Cell Cycle Dependent Agglutinability with Con A

The results in Table I suggested a correlation between the capacity of various treatments to reduce cytoagglutination and their effect on the distribution of cells over the cell cycle, viz. (partial) accumulation in the G_2 phase of the cell cycle.

Therefore, we started experiments to measure the agglutination of cells by Con A as a function of their position in the cell cycle. Previous results with Ebstein-Barr virus-transformed human lymphocytes suggested that the decrease in agglutinability started with

the initiation of DNA synthesis and reached a minimum at the end of this process when cells entered G_2 (Smets, 1973). In those cells the transition from low agglutinability of G_2 cells to high agglutinability of mitotic cells occured rather abruptly between late prophase and metaphase.

As shown in Table II, agglutinability of SV-3T3 cells decreases when cells pass from G_1 to G_2 and is maximal in mitotic cells. This decrease was measured more quantitatively by comparing the mid-point agglutination of SV-3T3 cells in G_2 phase as compared with asynchronous cells. G_2 phase cells showed a 6-7 fold decrease in agglutinability. Mid-point agglutination of G_2 cells was reached at a Con A concentration of 70-80 µg/ml, whereas the mid-point agglutination of asynchronous cells was reached with 10-15 µg/ml of Con A. Normal 3T3 cells showed mid-point agglutination at approx. 1000 µg/ml of Con A. In order to exclude the possibility that the decreased agglutinability was caused by a direct effect of X-rays on the cells, cells were grown in medium containing FUdr subsequent to X-irradiation. In these cultures cells were blocked at the entry of the S phase and not in G_2, and their agglutination was as high as in non-irradiated, FUdr treated controls. Apparently, the effect of irradiation on agglutinability was linked directly to its synchronizing effect. Similar cell cycle dependent changes in agglutinability have also been found in the spontaneously transformed 3T3-F line. The results in Table II demonstrate that at the concentration used, normal 3T3 cells are only highly agglutinable when in mitosis.

C. Distribution of Con A Binding Sites on the Cell Membrane

Because of this cell cycle dependent agglutinability of 3T3 and SV-3T3 cells, we investigated next whether the distribution of Con A binding sites on the cell surface was also dependent on the cell cycle. This was done by investigating HC-labeled cell bound Con A in replicas of normal and transformed 3T3 cells. It was hoped that this investigation might at the same time confirm results obtained in previous studies where normal and transformed 3T3 cells had been treated with Con A and several cytochemical markers. In those studies, we showed that on both cell types an irregular distribution of cell bound Con A was present on the cross sectioned plasma membrane (Collard and Temmink, 1974; Temmink and Collard, 1974; Temmink *et al.*, 1974). These results were in good agreement with those of de Petris *et al.* (1973), but seemed to be in conflict with those of other investigators (Nicolson, 1971; Huet and Bernhard, 1974; Garrido *et al.*, 1974).

When normal and transformed 3T3 cells were labeled with Con A and HC and the replicas of these cells were studied, the distribution of the labeled Con A on the membrane of 3T3 and SV-3T3 cells was irregular (Figs. 5A and B). Sometimes the irregularity of the distribution was less pronounced on SV-3T3 cells, because these

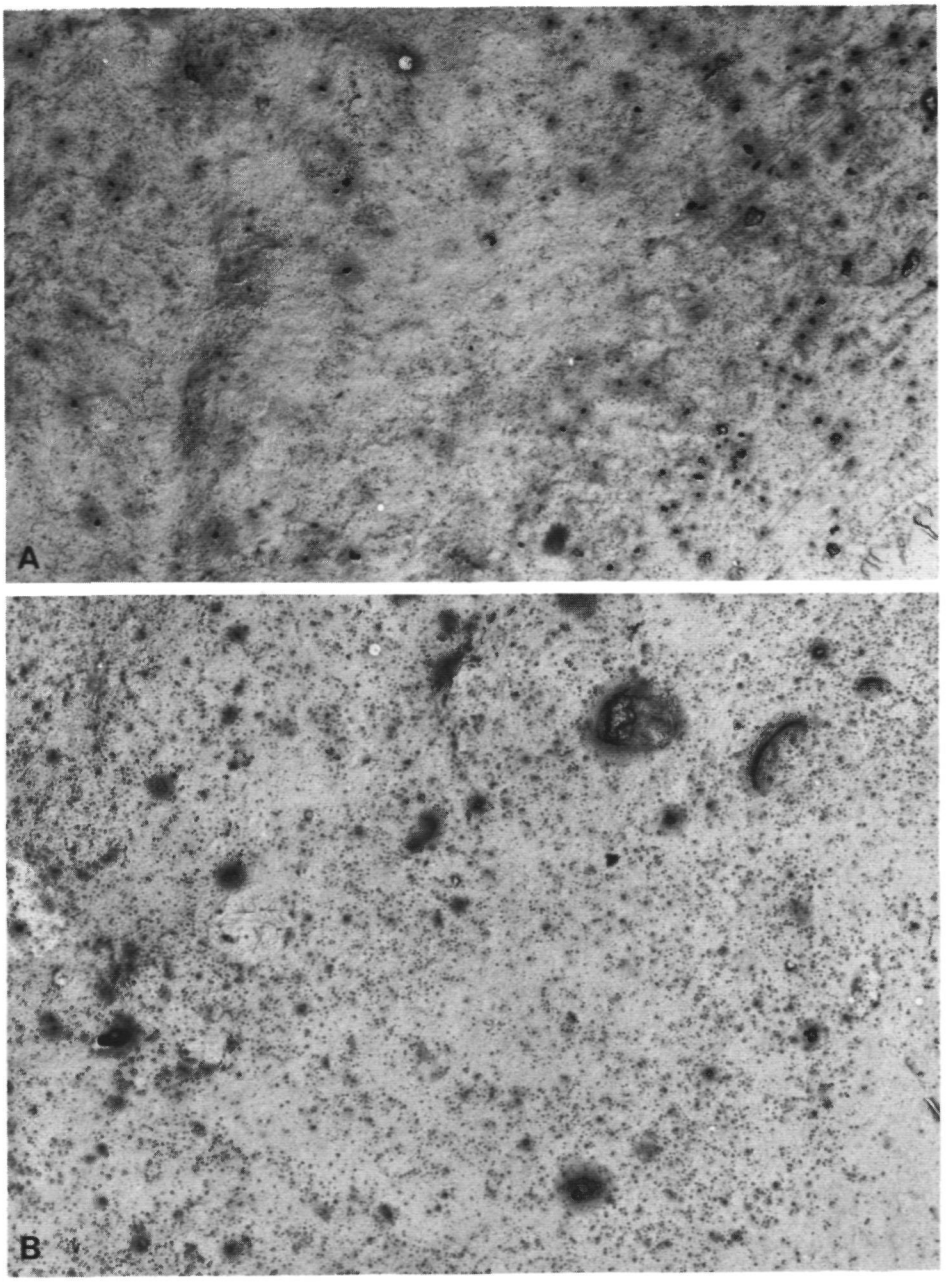

Fig. 5. Replica of HC-labeled mouse fibroblasts. Magn. 3,600 X. A. 3T3 cell; B. SV-3T3 cell.

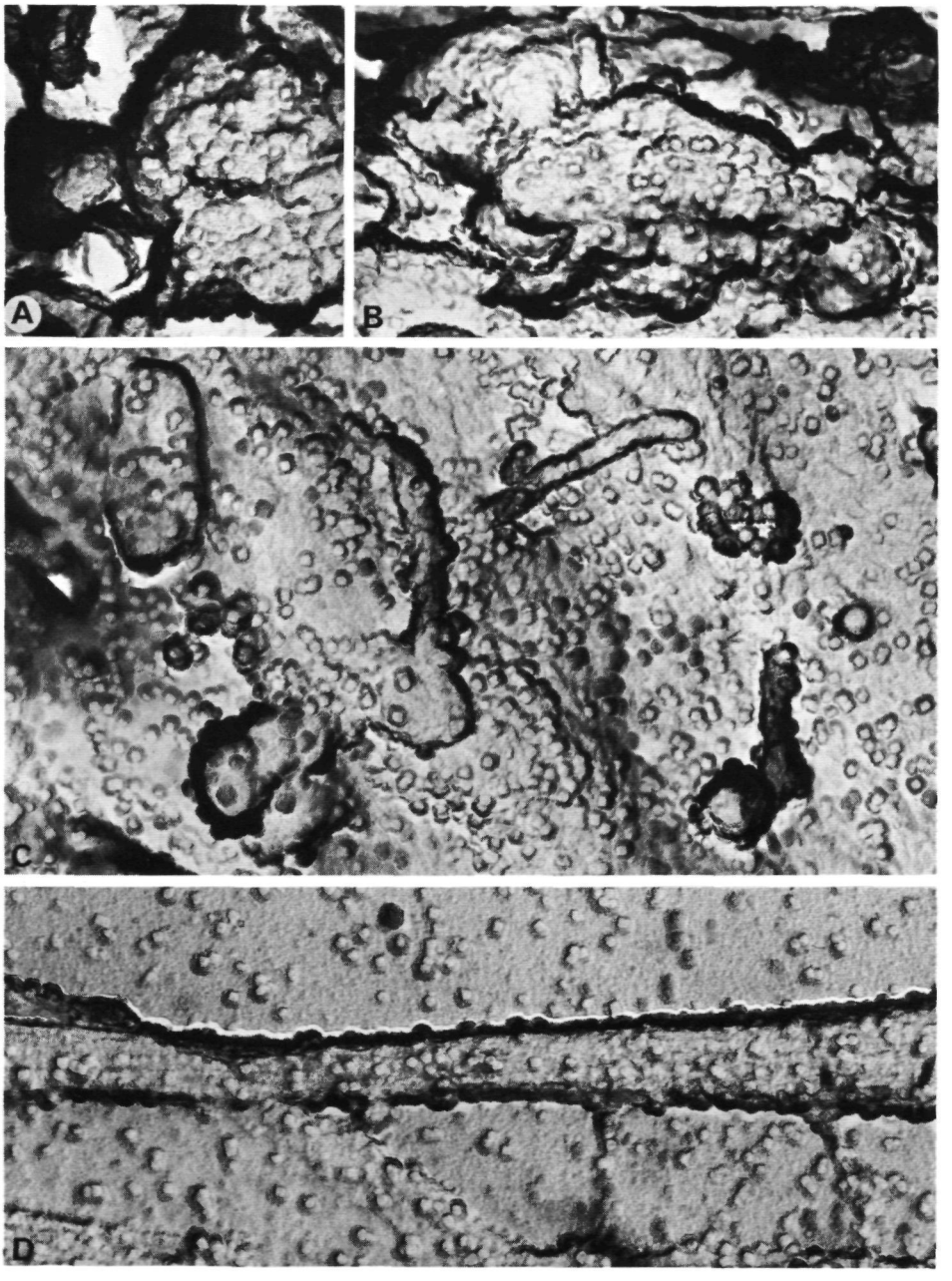

Fig. 6. Replica of parts of HC-labeled mouse fibroblasts. Magn. 45,000 X. A. Blebs on mitotic 3T3 cell; B. Blebs on mitotic SV-3T3 cell; C. Microvilli on SV-3T3 cell; D. Filopodium of 3T3 cell.

Fig. 7. Replica of HC-labeled 3T3 cell after cytokinesis. Magn. 3,400 X. HC molecules visible on some blebs, microvilli, and filopodia.

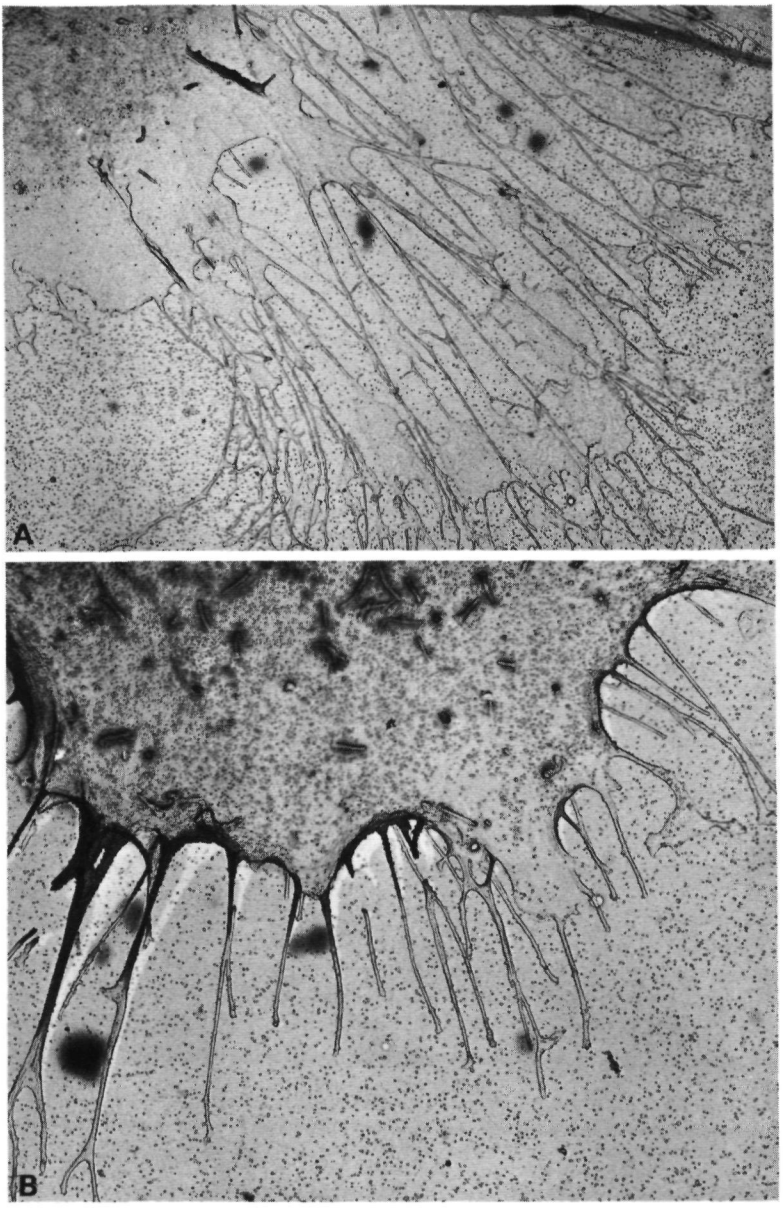

Fig. 8. Replica of lamellipodia of HC-labeled mouse fibroblasts. Magn. 3,600 X. A. 3T3 cell; B. SV-3T3 cell.

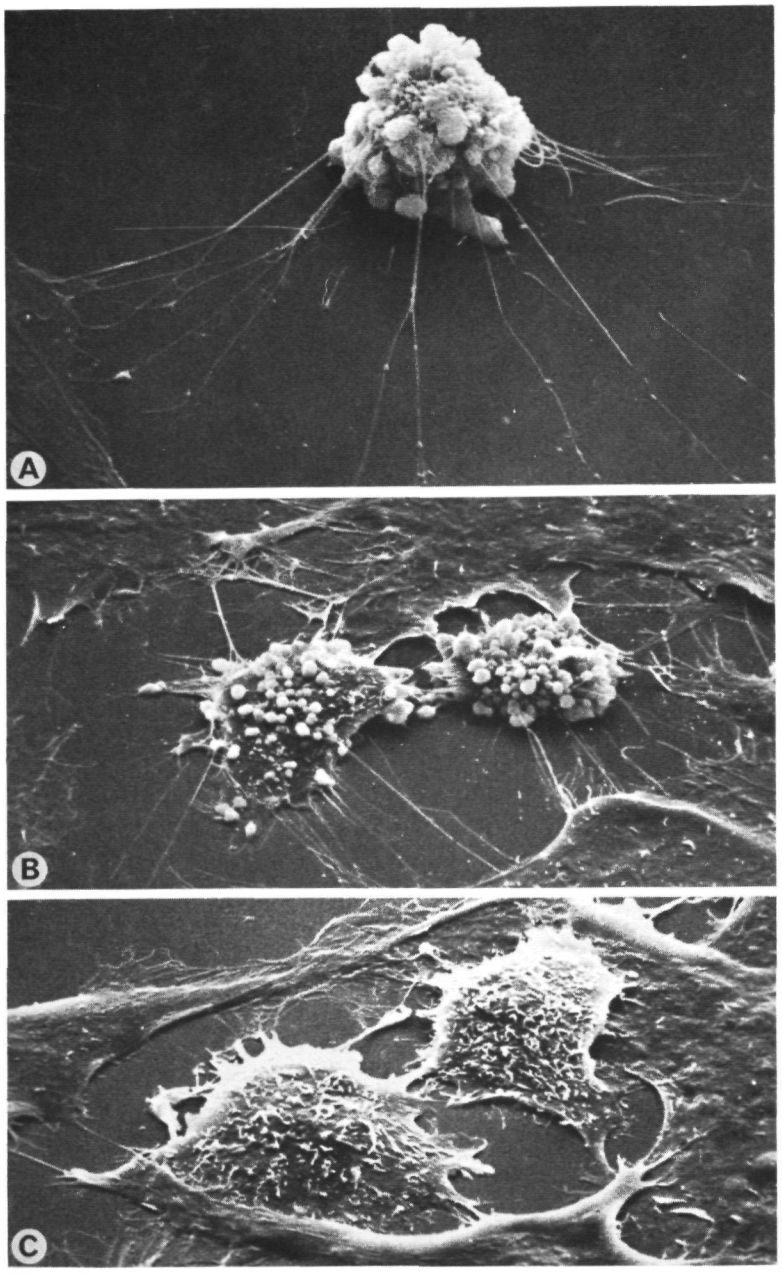

Fig. 9. Scanning electron micrographs of sub-confluent 3T3 fibroblasts *in situ*. Magn. 1,700 X. A. Cell in mitosis with filopodia attached to surface. B. Cells after cytokinesis with blebs and some microvilli. C. Cells in early G_1 with many microvilli.

cells generally had more HC-labeled Con A binding sites per unit surface area than 3T3 cells. The irregular distribution was found on most cells and over almost the complete cell surface, independent of the phase of the cell cycle. Thus, SV-3T3 cells in G_2 had the same distribution as 3T3 and SV-3T3 cells in other phases of the cell cycle. Blebs on cells in or shortly after mitosis (Figs. 6A and B, and 7) and microvilli on cells in early or middle G_1 (Fig. 6C) and even filapodia or retraction fibrils were covered with HC (Fig. 6D). However, there was one conspicuous difference between 3T3 and SV-3T3 cells. Normal 3T3 cells have very wide lamellipodia generally extending far from the nucleated center part of the cell body. These extensions were found to have very little HC-labeled Con A (Fig. 8A). In contrast, the lamellipodia of transformed cells were generally narrower and extended much less far from the cell body (Fig. 8B). These cellular extensions were often covered with more HC-labeled Con A than 3T3 lamellipodia, although with less HC than the rest of the cellular surface of SV-3T3 cells.

Thus the study of replicas of normal and transformed cells essentially confirmed our previously obtained data concerning the absence of differences in distribution of Con A binding sites between 3T3 and SV-3T3 cells. In addition, the study revealed differences in extension of the lamellipodia between normal and SV-3T3 cells and in detectable Con A binding sites on these lamellipodia. That these differences may have important implications for the explanation of the differences in agglutination between 3T3 and SV-3T3 cells will be discussed later.

D. Surface Morphology During the Cell Cycle

Our experiments had shown that the agglutinability of cells by Con A is cell cycle dependent and we confirmed in our study on replicas of HC-labeled cells that the differences in agglutination do not seem to be caused by differences in distribution of Con A binding sites between normal and transformed cells. Therefore it seemed worthwhile to investigate whether differences in agglutination could be correlated with cell cycle dependent morphological changes of the cells. 3T3 and SV-3T3 cells were prepared *in situ* for scanning electron microscopy and photographs were made of cells in different phases of the cell cycle.

Cells in S and G_2 were well spread over the cover slip and showed only very few surface projections. Upon entering mitosis dramatic morphological changes occured. The cells withdrew their lamellipodia and became rounded off (Fig. 9A). The surface became very irregular because of many blebs, and a number of filopodia retained contact with the glass surface. After mitotic division and cytokinesis (Fig. 9B) the two daughter cells gradually lost the surface blebs, but many microvilli were still visible (Fig. 9C). During G_1 the number of microvilli slowly decreased. Spreading of

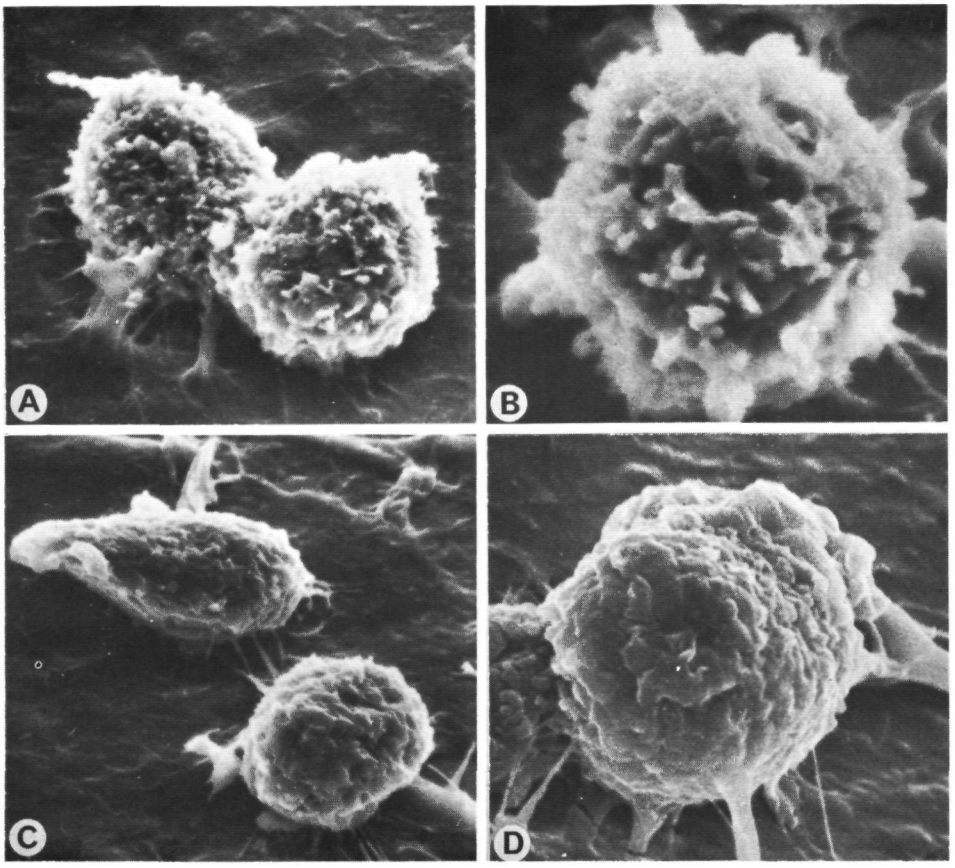

Fig. 10. Scanning electron micrographs of suspended fibroblasts as used in agglutination tests. The cells from confluent cultures were brought in suspension and, after prefixation, attached to a confluent layer of 3T3 cells. A. 3T3 cells, magn. 1,250 X; B. 3T3 cell, magn. 2,500 X; C. SV-3T3 cells, magn. 1,250 X; D. SV-3T3 cell, magn. 2,500 X.

the cells over the surface seemed to be guided by the old filopodia that had remained attached to the glass during mitosis.

The type of changes during the cell cycle was more or less the same for 3T3 and SV-3T3 cells. However the average degree of spreading was lower for SV-3T3 cells (confluent and superconfluent) than for 3T3 cells, and the morphological changes during the cell cycle were less pronounced in confluent cultures of transformed cells.

E. Surface Morphology of Suspended Cells

In order to correlate the surface morphology of normal and transformed cells with their difference in agglutinability by Con A, it is necessary to investigate the surface of these cells after they have been brought in suspension, as for agglutination assays. To that purpose we suspended normal and transformed cells with EDTA and used the same suspension for agglutination tests and for scanning electron microscopy of the surface.

Normal 3T3 cells, suspended with EDTA have a relatively rough surface with many protrusions and other irregularities (Fig. 10A and B). They also have a low agglutination with Con A (compare Table II). SV-3T3 cells, suspended with EDTA have a much more regular surface with less protrusions (Fig. 10C and D) and these cells have a high agglutination with Con A (compare Table II).

Thus there is a clear difference in morphological appearance between suspended normal and transformed cells, probably resulting from the phenotypic growth pattern in tissue culture.

IV. DISCUSSION

Several investigators have reported that drugs, such as dbc-AMP and protease inhibitors induce normal growth patterns in transformed cells (Hsie and Puck, 1971; Johnson *et al.*, 1971; Schnebli, 1972) and in addition decrease the agglutinability of these cells by plant lectins (Sheppard, 1971; Schnebli and Burger, 1972). Our results do not support this concept of reversion to normal growth behaviour (Smets, 1972; Collard and Smets, 1974). SV 40-transformed fibroblasts grown with these drugs accumulate in the G_2 phase of the cell cycle and not in G_1, as would have been expected if contact mediated growth had been induced (Figs. 1 - 4). The decrease in agglutinability correlates with a synchronization in the G_2 phase of the cell cycle (Table I). Our results on the effect of dbc-AMP on SV-3T3 cells were confirmed by Paul (1972). More recently Schnebli (Schnebli and Haemmerli, 1974) supported our conclusions on the effect of the protease inhibitor TLCK and indicated that previous suggestions of phenotypic reversion induced by TLCK had been premature.

In the present report we also demonstrate a clear cell cycle dependent agglutinability in synchronized, transformed cells (Table II). Cells in late S and G_2 have a low agglutination with Con A as compared to asynchronous cells or cells in early phases of the cell cycle. The synchronization procedures do not influence the described changes in agglutination during the cell cycle because we found a similar decrease in agglutinability of G_2 cells from an asynchronous culture (Smets and de Ley).

Normal cells in mitosis are highly agglutinable with the concentration of Con A used in this series. We are presently investigating variations in agglutinability during the cell cycle of normal cells with high concentrations of Con A to see whether cell cycle dependent agglutination is a common phenomenon or only specific for transformed cells.

Our results suggest that dbc-AMP and TLCK reduce the agglutination with Con A by synchronizing SV-3T3 cells in the G_2 phase of the cell cycle. However it cannot be excluded that these drugs alter the cells in such a way that they become less agglutinable and at the same time are arrested in G_2. Reduced motility of cells has been observed by us with both drugs (Smets, 1972; Collard and Smets, 1974) and could well explain the simultaneous occurrence of both phenomena in these cells. This would also imply that with other cells or with a different experimental approach dbc-AMP and TLCK might decrease agglutinability without concomitant synchronization of cells in G_2 phase.

When it became clear that differences in number of accessible Con A binding sites between normal and transformed cells could not sufficiently explain differences in agglutinability (Arndt-Jovin and Berg, 1971; Ozanne and Sambrook, 1971; Temmink and Collard, 1974), it was suggested that differences in distribution of Con A binding sites might be responsible for differences in agglutinability (Nicolson, 1971), correlated with cell transformation. Experiments on normal and transformed hamster cells seemed to support this suggestion (Bretton *et al.*, 1972; Martinez-Palomo *et al.*, 1972, Rowlatt *et al.*, 1973; Huet and Barnhard, 1974; Garrido *et al.*, 1974) but results on rat cells were more ambiguous (Bretton *et al.*, 1972; Garrido *et al.*, 1974) and investigations on murine fibroblasts apparently contradicted the assumed correlation between increase in clustering and in agglutinability (Smith, 1972; de Petris *et al.*, 1973). Our own previous investigations on 3T3 and SV-3T3 cells with different cytochemical markers (Collard and Temmink, 1974; Temmink and Collard, 1974; Temmink *et al.*, 1974) were in good agreement with those of de Petris *et al.* (1973) and Smith and Revel (1972). Because application of the replica technique on 3T3 and SV-3T3 cells seemed to lead to different results (Rosenblith *et al.*, 1973, Ukena *et al.*, 1974), we tried to confirm our previous results by using in the present study the replica technique also and we were able to confirm them. Thus we agree with Garrido *et al.* (1974) that results obtained with one cell system and transforming agent cannot be generalized for other cell systems and transforming agents. However, we feel that a more far-reaching conclusion should be drawn. The conflicting results indicate that differences in redistribution of lectin binding sites are not sufficient cause for differences in agglutination (Loor, 1973), and that increased membrane fluidity (Singer and Nicolson, 1972), as measured by lectin induced clustering, does not always coincide with transformation.

Whether uninduced, large scale differences in distribution of Con A binding sites between normal and transformed cells play a role in the agglutination of these cells remains to be established. We found less HC-labeled Con A binding sites on the lamellipodia of 3T3 cells than on SV-3T3 cells and are inclined to think that this might be partially responsible for differences in agglutinability. The results of Ukena *et al.* (1974) on secondary organization are, however, at variance with our results. Although this may simply result from the very low detection rate that they were able to achieve, additional experiments are required to settle this matter.

Our experiments on the effect of the dbc-AMP and protease inhibitors had shown that these reagents affect the cell cycle of transformed cells. In addition, it had been demonstrated that agglutinability of 3T3 and SV-3T3 cells is dependent on their position in the cell cycle. Therefore it seemed worthwhile to investigate whether cell cycle dependent changes in surface morphology could be detected that might influence the agglutination behavior. In Chinese hamster ovary cells, cell cycle dependent changes in surface morphology had been demonstrated recently (Rubin and Everhart, 1973; Everhart and Rubin, 1974). We found similar changes in mouse fibroblasts, and were able to confirm the phenotypic difference between 3T3 and SV-3T3 cells, as described by Porter *et al.*, (1973b). We thought that the morphological changes occuring during the cycle of normal and transformed cells might influence the agglutinability of these cells.

In order to obtain more evidence for the hypothesis that gross morphological differences (cell cycle dependent or otherwise) are important for differences in agglutinability, we started a number of new experiments. In the preliminary experiments described here, we were able to establish that morphological differences occur not only in attached cells spread on cover slips, but also remain detectable after detachment required for the agglutination tests. Additional experiments are needed to show that the morphological differences in detached cells do correlate with cell cycle dependent and transformation dependent phenotypic differences in cells growing on their substrate. Based on the information we have to date, we tentatively suggest that certain forms of transformation as well as the position in the cell cycle determine the morphology of growing cells and that this morphology is reflected in the detached cells used for agglutination tests. We suggest that these morphological differences between cells determine at least in part their difference in agglutination. More specifically, we are inclined to think that the lamellipodia of flattened cells appear as protrusions on the surface of cells following detachment from their substrate. Since these protrusions apparently contain less Con A binding sites and are more elaborate in normal cells, they might partly impair the agglutination response. In future experiments we hope to test this hypothesis.

REFERENCES

1. Anderson, T. F. (1951). "Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope." *Trans. N. Y. Acad. Sci. Ser.* II, 13, 130.

2. Arndt-Jovin, D. J., and Berg, P. (1971). "Quantitative binding of ^{125}I-concanavalin A to normal and transformed cells." *J. Virol.* 8, 716.

3. Bernhard, W., and Avrameas, S. (1971). "Ultrastructural visualization of cellular carbohydrate components by means of concanavalin A." *Exptl. Cell Res.* 64, 232.

4. Bretton, R., Wicker, R., and Bernhard, W. (1972). "Ultrastructural localization of concanavalin A receptors in normal and SV40-transformed hamster and rat cells." *Int. J. Cancer.* 10, 397.

5. Burger, M. M. (1970). "Proteolytic enzymes initiating cell division and escape from contact inhibition of growth." *Nature* 227, 170.

6. Burger, M. M. (1971). "The significance of surface structure changes for growth control under crowded conditions. In: *Growth control in cell cultures* (Ed. G. E. Wolstenholme) p. 45. Churchill-Livingstone, London.

7. Collard, J. G., and Smets, L. A. (1974). "Effect of proteolytic inhibitors on growth and surface architecture of normal and transformed cells." *Exptl. Cell Res.* In press.

8. Collard, J. G., and Temmink, J. H. M. (1974). "Binding and cytochemical detection of cell-bound concanavalin A." *Exptl. Cell Res.* In press.

9. Comoglio, P. M., and Guglielmone, R. (1972). "Two-dimensional distribution of concanavalin A receptor molecules on fibroblast and lymphocyte plasma membranes." *FEBS Lett.* 27, 256.

10. De Petris, S., Raff, M. C., and Mallucci, L. (1973). "Ligand-induced redistribution of concanavalin A receptors on normal, trypsinized and transformed fibroblasts." *Nature New Biology* 244, 275.

11. Everhart, L. P., and Rubin, R. W. (1974). "Cyclic changes in the cell surface. II. The effect of cytochalasin B on the surface morphology of synchronized chinese hamster ovary cells." *J. Cell Biol.* 60, 442.

12. Garrido, J., Burglen, M-J., Samolyk, D., Wicker, R., and Bernhard, W. (1974). "Ultrastructural comparison between the distribution of concanavalin A and wheat germ agglutinin cell surface receptors of normal and transformed hamster and rat cell lines." *Cancer Res.* 34, 230.

13. Hsie, A. W., and Puck, T. T. (1971). "Morphological transformation of chinese hamster cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone." *Proc. Nat. Acad. Sci. U. S. A.* 68, 358.

14. Huet, Ch., and Bernhard, W. (1974). "Differences in the surface mobility between normal and SV40-, polyoma- and adenovirus-transformed hamster cells." *Int. J. Cancer* 13, 227.

15. Inbar, M., and Sachs, L. (1969). "Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells." *Proc. Nat. Acad. Sci. U. S. A.* 63, 1418.

16. Inbar, M., Ben-Bassat, H., and Sachs, L. (1972). "Membrane changes associated with malignancy." *Nature New Biology* 236, 3.

17. Johnson, G. S., Friedman, R. M., and Pastan, I. (1971). "Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3':5'-cyclic monophosphate and its derivative." *Proc. Nat. Acad. Sci. U. S. A.* 68, 425.

18. Loor, F. (1973). "Lectin-induced lymphocyte agglutination." *Exptl. Cell Res.* 82, 415.

19. Martinez-Palomo, A., Wicker, R., and Bernhard, W. (1972). "Ultrastructural detection of concanavalin A surface receptors in normal and in polyoma-transformed cells." *Int. J. Cancer* 9, 676.

20. Nicolson, G. L. (1971). "Difference in topology of normal and tumour cell membranes as shown by different surface distributions of ferritin conjugated concanavalin A." *Nature New Biology* 233, 244.

21. Nicolson, G. L., and Singer, S. J. (1971). "Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy: Application to saccharides bound to cell membranes." *Proc. Nat. Acad. Sci. U. S. A.* 68, 942.

22. Ozanne, B., and Sambrook, J. (1971). "Binding of radioactively labeled concanavalin A and wheat germ agglutinin to normal and virus-transformed cells." *Nature New Biology* 232, 156.

23. Paul, D. (1972). "Effects of cyclic AMP on SV-3T3 cells in culture." *Nature New Biology* 240, 179.

24. Porter, K., Prescott, D., and Frye, J. (1973a). "Changes in surface morphology of chinese hamster ovary cells during the cell cycle." *J. Cell Biol.* 57, 815.

25. Porter, K. R., Todaro, G. J., and Fonte, V. (1973b). "A scanning electron microscope study of surface features of viral and spontaneous transformants of mouse Balb/3T3 cells." *J. Cell Biol.* 59, 633.

26. Rosenblith, J. Z., Ukena, T. E., Yin, H. H., Berlin, R. D., and Karnovsky, M. J. (1973). "A comparative evaluation of the distribution of concanavalin A binding sites on the surfaces of normal, virally-transformed, and protease-treated fibroblasts." *Proc. Nat. Acad. Sci. U. S. A.* 70, 1625.

27. Rowlatt, C., Wicker, R., and Bernhard, W. (1973). "Ultrastructural distribution of concanavalin A receptors on hamster embryo and adenovirus tumour cell cultures." *Int. J. Cancer* 11, 314.

28. Rubin, R. W., and Everhart, L. P. (1973). "The effect of cell-to-cell contact on the surface morphology of chinese hamster ovary cells." *J. Cell Biol.* 57, 837.

29. Schnebli, H. P., and Burger, M. M. (1972). "Selective inhibition of growth of transformed cells by protease inhibitors." *Proc. Nat. Acad. Sci. U. S. A.* 69, 3825.

30. Schnebli, H. P., and Haemmerli, G. (1974). "Protease inhibitors do not block transformed cells in the G1 phase of the cell cycle." *Nature* 248, 150.

31. Sheppard, J. R. (1971). "Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3':5'-cyclic monophosphate." *Proc. Nat. Acad. Sci. U. S. A.* 68, 1316.

32. Singer, S. J., and Nicolson, G. L. (1972). "The fluid mosaic model of the structure of cell membranes." *Science* 175, 720.

33. Smets, L. A. (1972). "Contact inhibition of transformed cells incompletely restored by dibutyryl cyclic-AMP." *Nature New Biology* 239, 123.

34. Smets, L. A. (1973). "Agglutination with Con A dependent on cell cycle." *Nature New Biology* 245, 113.

35. Smets, L. A., and de Ley, L. "Cell cycle dependent modulations

of the surface membrane of SV40-transformed 3T3 cells." Submitted for publication.

36. Smith, S. B., and Revel, J-P. (1972). "Mapping of concanavalin A binding sites on the surface of several cell types." *Dev. Biol.* 27, 434.

37. Stobo, J. D., and Rosenthal, A. S. (1972). "Biologically active concanavalin A complexes suitable for light and electron microscopy." *Exptl. Cell Res.* 70, 443.

38. Temmink, J. H. M., and Collard, J. G. (1974). "Correlation between cytochemical detection by horse-radish peroxidase and binding of concanavalin A on normal and transformed mouse fibroglasts." In: *Electron microscopy and cytochemistry*, p. 299. (Eds. E. Wisse, W. Th. Daems, I. Molenaar, and P. van Duyn) North-Holland, Amsterdam.

39. Temmink, J. H. M., Collard, J. G., Spits, H., and Roos, H. (1975). "A comparative study of four cytochemical detection methods of concanavalin A binding sites on the cell membrane." Submitted for publication.

40. Terasima, A., and Tolmach, L. J. (1963). "Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells." *Exptl. Cell Res.* 30, 344.

41. Ukena, T. E., Borysenko, J. Z., Karnovsky, M. J., and Berlin, R. D. (1974). "Effects of colchicine, cytochalasin B, and 2-deoxyglucose on the topographical organization of surface-bound concanavalin A in normal and transformed fibroblasts." *J. Cell Biol.* 61, 70.

Paper III

Experimental Cell Research 95 (1975) 95–102

CONTACT-MEDIATED CHANGES IN CYTOAGGLUTINATION

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SUMMARY

Cytoagglutination with Concanavalin A was studied in SV3T3 cells as a function of cell density. Agglutinability was low in subconfluent cultures (midpoint concentration 200 μg/ml) buth high in multilayered cultures (midpoint concentration 10–15 μg/ml) Normal 3T3 cells retained low agglutinability (midpoint concentration 1000 μg/ml) even when seeded at superconfluent density. By growing SV3T3 cells at low and at high density in the same culture dish it could be excluded that density modulation of cytoagglutination was caused by differences in pH or nutrient supply Changes in the density of ConA binding sites or in ATP concentration could not account for the 20-fold difference in agglutinability between cells from high and low density regions Cell kinetic studies demonstrated that all cells in high and low density cultures were in log phase of growth, differing only in the amount of intercellular contact. In Py-BHK cells, density modulation of agglutinability was much less demonstrated. Unlike SV3T3 cells, these cells rearranged on the substrate when seeded at low density to form clusters of cells with intensive overlapping contact The results suggest that in transformed cells, cell-to-cell contact is a major determinant of high agglutinability which therefore seems the result, rather than the cause, of uncontrolled growth

Proliferation of untransformed cells is controlled by mechanisms which require mutual cell contact (contact inhibition of movement [1] and contact or topoinhibition of growth [2, 3]). By consequence, defective growth control in transformed cells is first manifested by the absence of these regulatory mechanisms when cells are in mutual contact.

In addition, transformed cells are characterized by increased agglutination with the plant lectins Concanavalin A (ConA) and wheat germ agglutinin (WGA) which has been correlated with the loss of growth control [4, 5] and with tumorigenicity [6, 7].

These observations may suggest that in transformed cells the surface changes causing increased agglutinability are also related to—if not identical with—the membrane alterations responsible for defective growth control. However, it is by no means excluded that increased cytoagglutination is a casual or secondary consequence of malignant transformation or even the result, rather than the cause, of uncontrolled growth.

The question as to the role of the condition of high agglutinability in the breakdown of growth control might find an answer by studying cytoagglutination as a function of cell contacts as these are obviously involved in growth control.

In a number of papers, modulations of agglutinability of transformed cells by variations in cell density have been reported [8–11]. However, none of these studies gave conclusive evidence that variation in the amount of intercellular contact was the

most important factor in the observed effects. Rather, it has been concluded [11] that density-dependent modulations of agglutination were caused by fluctuations in cellular ATP content induced by culture age. Moreover, cells grown at various densities may differ in their proliferative capacity (i.e. the ratio of log and plateau phase cells versus cycling cells) and in the distribution of cells over the various phases of the cell cycle, both conditions known to affect cytoagglutination (see refs [9] and [12] respectively).

Therefore, to investigate the possible role of cell contact in cytoagglutination by comparing cells grown at different densities, the contribution of factors such as nutrient supply and cell kinetics should be carefully checked.

In this paper we wish to present data suggesting that cell contact is a major factor in the expression of high agglutinability with ConA in transformed fibroblasts.

MATERIALS AND METHODS

Cell culture

Mouse 3T3 cells and SV40 virus transformed cells (SV3T3) were obtained commercially from Flow Laboratories and Py-BHK cells were obtained by courtesy of Dr Allen, Newcastle The cell lines were grown as previously described [12] in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum in plastic dishes of 5 or 10 cm diameter filled with 5 or 10 ml of medium, respectively

Growth at different densities

To obtain regions of high and low cell density in the same culture dish, a concentrated cell suspension, prepared by trypsinization, was added to a dish which was held in a tilted position over 30° The cells were allowed to attach for 20–30 min at superconfluent density in a segment covering about 20% of the surface and its boundary was marked at the outside of the dish Next, the dishes were placed in horizontal position, the medium of the first inoculum was removed and 5 ml of a diluted suspension was added to obtain a region of low density in the remaining free surface of the dish The cell numbers in the inocula were chosen to obtain half-confluent density in the sparse region and a 4–5-fold higher density in the dense region of the dish In this way, the cultures contained equal numbers of cells grown at either high or low density Seeding at superconfluent density did not affect the viability of 3T3 cells during the 24 h of the experiment

Agglutination and binding of ^{3}H-ConA

To suspend the cells for agglutination and binding assays, the cultures were washed 3 times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and incubated for 1 min in 5 ml sodium versenate (5×10^{-5}M) The versenate was next sucked from the dishes which were incubated at 37°C for 5–10 min The cells were suspended in ice-cold, serum-containing culture medium for 10 min

The resulting suspension was counted in a haemocytometer and assayed for viability by trypan blue exclusion Only suspensions of essentially single cells with over 90% viability were used in the assays Samples containing 2×10^6 cells were washed with PBS and resuspended in 1 ml of ConA in PBS Control assays without ConA were always included to control for aspecific agglutination Agglutination was performed as described earlier [13] and scored after 20 min in the usual scale (0–++++)

A crude preparation of generally labelled ^{3}H-ConA was obtained commercially and purified by centrifugation and chromatography on Biogel P-100 The purified product (spec act 2000 dpm/μg) was undistinguishable from unlabelled ConA (Calbiochem, A grade) in agglutination experiments and in chromatographic behaviour Binding of ConA was assayed by suspending the cells at 0°C in 50 μg ^{3}H-ConA/ml of PBS Aliquots of the suspension were sampled on filter disks, washed and counted after several incubation times Control for specificity of the binding were performed by inclusion of α-methyl-D-glucoside in the reaction mixture The amount of ^{3}H ConA bound in 20 min corresponded approximately with the saturation level of rapidly labelled binding sites

Cell cycle kinetics

Cell numbers were determined with the haemocytometer and some fields of the haemocytometer were photographed to determine cell diameters Distribution of cells over the various phases of the cell cycle was measured by pulse-cytophotometry of DNA fluorescence following staining with ethidium bromide using the ICP 11 impulse cytophotometer (Phywe AG, Gottingen)

Details of cell preparation and staining methods have been published [14] The assay of the fraction of cells capable of traversing the cell cycle by measuring the accumulation in G2 phase following X-ray treatment has been described [15]

ATP determination

The amount of ATP per cell or per mg of cell protein was measured by the luciferin-luciferase assay as described by Vlodavsky et al [11]

Table 1. *Effect of subculture on agglutination and ATP content in SV3T3 cells*

Time after subculture (hours)	Cell density (cells/cm^2 $\times 10^5$)	Agglutination (20 min; 20 μg/ml)	ATP content (mol/cell $\times 10^{-5}$)	ATP content (mol/mg protein $\times 10^{-8}$)
0[a]	2.7	++++	0.07	0.01
3	0.2	++	0.7	0.14
5	–[b]	+(+)	–	–
6	–	0	–	–
24	0.4	+	2 2	0 63
48	1 2	++	–	–
72	3 0	++++	0 06	0.01

Cells were subcultured from suspensions made with sodium versenate as described in 'Materials and Methods' The values are from three independent experiments.

[a] Stock culture.

[b] Not determined

RESULTS

Effect of culture age on agglutination and ATP content

SV3T3 cells from dense stock cultures were suspended with sodium versenate. Parts of the suspension were assayed for agglutination with ConA or for cellular ATP content. The remaining cells were used to inoculate culture dishes at low density which were assayed after various hours of growth. Table 1 shows that ConA agglutinability decreased rapidly in the first hours of subculture with a parallel increase in ATP content. During further growth of the cultures, high agglutinability was gradually reassumed and the ATP content dropped to low values again.

The results indicate that agglutinability is inversely proportional to ATP content as described by others [11] and correlates with cell density or culture age [8, 9].

Table 2 shows that cells from dense stock cultures plated at 10-fold dilution lose their agglutinability within 6 h whereas cells plated under superconfluent conditions did not do so, or to a lesser degree. Moreover, cytoagglutination of sparse cells replated at 5-fold higher density increased rapidly. The results demonstrate at least qualitatively that the successive removals with versenate did not per se account for the loss of cytoagglutination in sparse cells. Moreover, they suggest that altered cell density, rather than conditions of suspending and replating, were mainly responsible for the observed effects.

Table 2. *The effect of plating density on agglutination with ConA (20 μg/ml) of SV3T3 cells*

Hours after plating	Dense cells (2.5×10^5/cm^2) plated at low density (0.25×10^5/cm^2)	Dense cells (2.5×10^5/cm^2) plated at high density ($1\ 5\times10^5$cm^2)	Sparse cells (0.30×10^5/cm^2) replated at high density ($1\ 5\times10^5$/cm^2)
0[a]	++++	++++	+
6	0	+++	+++(+)
24	+	+++(+)	n d

[a] Agglutination of versenate suspended cells before plating.

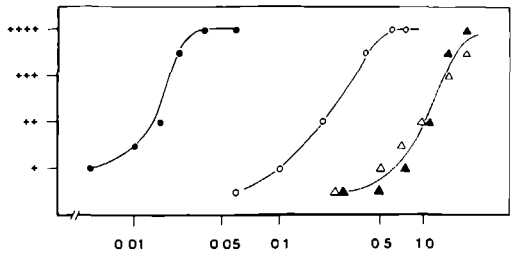

Fig 1 Abscissa agglutination response, *ordinate* ConA conc (mg/ml) SV3T3 cells grown at ●—●, high density ($1\ 7\times10^5$ cells/cm²), ○—○, low density ($0\ 40\times10^5$/cm²), 3T3 cells grown at △—△, high density ($1\ 6\times10^5$/cm²), ▲—▲, low density ($0\ 35\times10^5$/cm²)

Agglutination with ConA of SV3T3 and 3T3 cells grown at high and low cell density in the same culture dish

Effect of local cell density

To better discriminate between the effect of cell density and culture age on agglutination, normal 3T3 and SV3T3 cells were grown at low and high density in the same culture dish as described under 'Materials and Methods'. After 24 h, the cells from either the sparse or the dense regions were scraped off the dish and discarded. The remaining dense or sparse regions were then suspended with sodium versenate as described under 'Materials and Methods' and used in the agglutination or the binding assays.

Fig. 1 shows the agglutination with various ConA concentrations. Agglutination of 3T3 cells was low (half-maximal agglutination concentration 1 000 μg/ml) for both subconfluent and superconfluent cells. However, a 15–20-fold difference in agglutinability was observed in superconfluent SV3T3 cells (half-maximal agglutination concentration 10–15 μg/ml) as compared with subconfluent cells (half-maximal agglutination concentration 200 μg/ml). These results indicate that cell density specifically promotes the ConA agglutinability of the transformed cells.

Table 3 shows the effect of local cell density on ATP content, cell volume and binding of ^{3}H-ConA. SV3T3 cells from a dense region were much smaller than the cells from a sparse region as measured in the suspension used for the agglutination assay. This voluminal change was much less expressed in 3T3 cells. Dense SV3T3 cells bound less ConA than did cells grown

Table 3. *Effect of local cell density on agglutination, cell size, binding of ^{3}H-ConA and ATP content in 3T3 and SV3T3 cells grown for 24 h at low or high cell density*

	3T3		SV3T3	
	Low density	High density	Low density	High density
Cell density (cells/cm² × 10^5)	0 35	1 60	0 40	1 7
Relative cell volume	1 00	0 80	1 00	0 58
Agglutination[a] (μg ConA/ml)	1 000	800–1 000	200	10–15
^{3}H-ConA[b] (μg/10^6 cells)	1 95	1 86	2 73	1 92
ATP content				
mol/cell × 10^{-15}	2 5	1 3	1 9	1 1
mol/mg protein × 10^{-8}	0 69	0 57	0 75	0 51

Cells were collected separately from sparse and dense regions of the culture dish as described in 'Materials and Methods' Values from three independent experiments

[a] Lectin concentration for half-maximal agglutination in 20 min

[b] Amount bound in 20 min at 0°C

at low density but in 3T3 cells no such variations in lectin binding were observed. Both normal and transformed 3T3 cells contained less ATP per cell when grown at superconfluent density. These differences were less manifest when calculated per mg of protein and almost negligible when calculated per unit of cell volume.

It is concluded from these results that low and high density SV3T3 cells did not differ substantially in the concentration of cellular ATP nor in the number of ConA binding sites per unit of cell surface.

Incubation in 0.001 % trypsin for 5 min at room temperature prior to the agglutination assay, stimulated the agglutinability of low density SV3T3 cells to the level of high density cells without causing aspecific agglutination in controls incubated without the lectin. This response to proteolytic treatment is comparable to that of untransformed 3T3 cells [16].

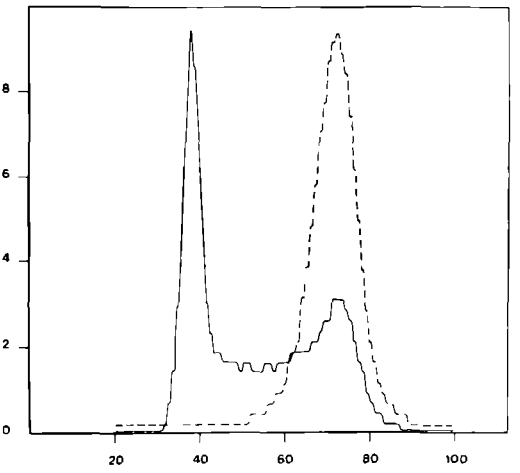

Fig. 2. Abscissa no. of cells ($\times 10^{-3}$), *ordinate*. channel no. —, Low density SV3T3 cells (0.25×10^5/cm^2) 24 h after subculture, ---, low density SV3T3 cells irradiated with 1200 R X-rays at 24 h after subculture and analysed 24 h later.

DNA per cell distribution, represented by channel number, of SV3T3 cells grown at low density for 24 h and of the same cells grown for an additional 24 h following X-irradiation Channel 38 mean DNA content of G 1 phase cells, channel 76 mean DNA content of G 2 phase cells, channels 40–70. range of S phase cells

Cell cycle kinetics and agglutinability

To check the cell cycle effects on agglutinability in low density cultures (compare refs [12] and [17]), the DNA per cell distribution was measured in sparse cultures of SV3T3 cells at 24 h after seeding. Fig. 2 (solid graph) shows that these cultures contained cells with a DNA per cell distribution characteristic for asynchronous growth. To confirm that these cells were actually traversing the cell cycle, sparse cultures were irradiated and the DNA per cell distribution was recorded 24 h thereafter. As demonstrated by fig. 2 (dashed graph) virtually all irridiated cells became arrested at the radiation-induced block in G2 phase indicating that cells of sparse cultures were capable of cell cycle traverse.

Similar results as to cell cycle distribution and cell cycle traverse have been reported for SV3T3 cells grown at high density [12]. Consequently, the sparse as well as the dense cells used in the present experiments are all cells in logarithmic phase of growth, differing only in the amount of intercellular contact.

The cell cycle dependent changes in agglutinability reported for multilayered SV3T3 cells [12] were not observed in subconfluent cultures. G2 arrested cells and asynchronous controls (fig. 2) showed the same low agglutinability with ConA.

Agglutination and cell arrangement on substrate

To further investigate the role of cell contact on cytoagglutination with ConA, density modulation of agglutinability was studied in correlation with the amount of cell contact in sparse cultures.

Table 4. *Density modulation of agglutination and the degree of cell contact*

Cells	Time after plating (hours)	ConA (μg/ml) for half-maximal agglutination		% Sparse cells making[c]		
		High density[a]	Low density[b]	no contact	lateral contact	overlapping contact
3T3	24	1 000	1 000	60	40	0
SV3T3	24	10–15	200	20	70	10
Py-BHK	6	5	50	65	25	10
Py-BHL	24	4–5	10–15	5	30	65

Versenate suspended cells were plated at either low or high density and assayed 6 or 24 h thereafter
[a] Approx 2.0×10^5 cells/cm^2
[b] Approx. 0.25×10^5 cells/cm^2.
[c] See text.

To this end, 3T3, SV3T3 and Py-BHK cells were seeded at low density and the cells were fixed and stained in situ after 2, 6 and 24 h, respectively. The amount of cell contact in the preparations was scored as the percentages of free cells, cells in lateral contact and cells with intensive overlapping contact. In cultures fixed 2 h after seeding, the nuclei of all cell lines were randomly distributed on the substrate with only incidental contact by poorly stretched cells.

As shown in table 4, density-dependent modulation of agglutinability was much less expressed in 24-h-old cultures of Py-BHK cells as compared with SV3T3 cells. The former cells had rearranged in 24 h of culture to form clusters of 5–8 cells with considerable nuclear and cytoplasmic overlap. However, a much lower degree of cell–cell contact and of cytoagglutination was observed in sparse Py-BHK cells assayed 6 h after plating. Partial rearrangement of SV3T3 cells had occurred as well but most of these cells made only lateral contact with their neighbours. Lateral contact in 3T3 cells did not result from nuclear displacement but from extensive spreading of the cytoplasm.

The results confirm the postulate that the decrease in agglutinability in sparse SV3T3 cells is primarily due to diminished intercellular contact.

DISCUSSION

According to a recent review by Nicolson [18], cytoagglutination is the final outcome of a great many promoting as well as inhibitory factors. By consequence, the effects of cell density on ConA agglutinability reported here and by others [8–10] need not a priori to be paralleled by qualitative and quantitative similar changes in cytoagglutination with other lectins (compare ref. [10]). Similarly, the effects of cell density are not necessarily mediated by variations in the amount of cellular contact but can equally well result from secondary consequences of high cell density.

However, the results in table 3 have demonstrated that variation in ATP concentration is not a necessary condition in density dependent modulation of agglutinability as suggested by others [11]. The technique of growing cells at different densities in the same culture dish excluded also that variation in nutrient supply, accumulation of metabolites or differences in pH were responsible for the observed effects. Moreover, decreased agglutinability

of low density SV3T3 cells could not be ascribed to unusual distribution over the cell cycle phases nor to the presence of detectable numbers of non-cycling cells (fig. 2). For this reason, our culture system is not comparable with SV3T3 cells at much lower density [10] or with 3T6 cells in plateau phase [9] in which somewhat different effect on agglutination have been observed. Finally, normal 3T3 cells seeded at superconfluent density were prepared for the agglutination assay with great difficulty but their agglutinability did not differ from that of cells from subconfluent cultures (fig. 1). This observation invalidates the possible argument that the disruption of multilayered cell sheets per se accounts for the high agglutinability of transformed cells in dense cultures.

Thus, since neither metabolic condition, cell cycle stage, nor physical stress can explain the effect of cell density on cytoagglutination with ConA, the conclusion is that transformed cells require intensive cell contact for maximal expression of their characteristic high agglutinability. The apparent correlation between the amount of cell–cell contact and of agglutinability in Py-BHK cells (table 4), the low agglutinability of flat mutants of SV3T3 cells which do not overgrow each other [19] and the acquisition of high agglutinability of mouse lymphosarcoma cells when grown in standing (but not in agitated) suspension cultures [13], are in support of this conclusion.

Low agglutination of sparse SV3T3 cells cannot be ascribed to a decrease in ConA binding sites per unit of cell surface (table 3). On the other hand, cell stretching and increased cell volume may be instrumental in this response since similar changes in cell morphology have been observed to accompany the decrease in cytoagglutination during cell cycle traverse [17, 20]. Moreover, the absence of cell cycle effects in sparse cultures reported here strongly suggests that the effect of diminished contact on cell morphology had dominated over similar changes occurring in the cell cycle.

Since it appears that in the two transformed cell lines studied, cytoagglutination is maximal in a situation of considerable overlapping contact—that is, a situation resulting from defective contact inhibition of movement or of growth—high agglutinability of these cells seems to some extent to be the result rather than the cause of uncontrolled growth. However, the results also demonstrate that intensive intercellular contact stimulates only the agglutinability of the transformed cells. These observations strongly suggest that SV3T3 cells can mutually modify their contacting surfaces by specific interactions. These interactions may be identical with the enzymic modification of the cell periphery as a regulatory factor in cell growth as discussed among others by Poste [21].

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REFERENCES

1. Abercrombie, M & Heaysman, J E, Exp cell res 6 (1954) 293
2. Stoker, M G & Rubin, H, Nature 215 (1967) 171
3. Dulbecco, R, Nature 227 (1970) 802
4. Burger, M, Fed proc 32 (1973) 91
5. Pollack, R E & Burger, M, Proc natl acad sci US 62 (1969) 1074
6. Aaronson, S A & Todaro, G J, Science 162 (1968) 1024
7. Inbar, M, Ben-Bassat, H & Sachs, L, Nature new biol 236 (1972) 3
8. — Proc natl acad sci US 68 (1971) 2748
9. Goto, M, Kataoka, Y, Goto, K, Yodoyama, T & Sato, H, Gann 63 (1972) 505
10. Nicolson, G & Lacorbiere, M, Proc natl acad sci US 70 (1973) 1672.
11. Vlodavsky, I, Inbar, M & Sachs, L, Proc natl acad sci US 70 (1973) 1780.
12. Smets, L A & De Ley, L, J cell physiol 84 (1974) 343.
13. Smets, L A & Broekhuysen-Davies, J, Eur J cancer 8 (1972) 541

14. Smets, L A, Nature new biol 239 (1972) 123.
15. Collard, J G & Smets, L A, Exp cell res 86 (1974) 75.
16. Burger, M, Proc natl acad sci US 62 (1969) 994.
17. Smets, L A, Nature new biol 245 (1973) 113.
18. Nicolson, G, Int rev cytol 39 (1974) 89.
19. Smith, H, Hiller, A, Kingsberg, E & Roberts-Dory, C, Nature new biol 245 (1973) 67.
20. Porter, K, Prescott, D & Frye, J, J cell biol 57 (1973) 815.
21. Poste, G, Exp cell res 73 (1972) 319.

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Paper IV

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BINDING AND CYTOCHEMICAL DETECTION OF CELL-BOUND CONCANAVALIN A

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SUMMARY

We have investigated the relationship between the total amount of cell-bound concanavalin A (con A), as determined in binding experiments with ^{3}H-con A, and the amount of cell-bound con A detected with horseradish peroxidase on normal murine fibroblasts (3T3) By comparing prefixed and non-prefixed cell membranes a discrepancy was found between the amount of cell-bound con A and the amount of cytochemically detected con A This discrepancy was interpreted to substantiate the theory that con A binding sites can move within the membrane Incubation of non-prefixed cells with con A induced redistribution of binding sites on the cell membrane The redistribution resulted in changes in detectability of con A by horseradish peroxidase The use and limitations of horseradish peroxidase in the study of cell transformation and of changes in agglutinability by con A are discussed

The plant lectin concanavalin A (con A) is known to bind to the cell membrane of animal cells and preferentially agglutinate transformed cells [1] In addition, increased agglutinability by plant lectins is correlated with increased tumorigenicity [2] and loss of growth control in vitro [3, 4] The plasma membrane is thought to play an important role in these phenomena and con A can be detected in the electron microscope by a number of cytochemical techniques [5, 6, 7] Con A therefore seemed an ideal tool for the study of changes in the ultrastructure of the plasma membrane as a result of transformation

Results of others [2, 8, 9] and our own [10] indicate that changes in the agglutinability are not always correlated with changes in the amount of cell-bound con A per unit surface area It was thought, therefore, that changes in topographical distribution or expression of con A binding sites might play an important role in the agglutination process

In our experiments [10] concerning differences between normal and transformed murine fibroblasts (3T3) a discrepancy occurred between the amount of cell-bound ^{3}H-con A and the amount of con A detected with horseradish peroxidase (HRP) under the same circumstances The amount of ^{3}H-con A bound to the cells in 15 min was considered to be equal to the amount of non-tritiated con A that the cells would have bound in the same time Thus the amount of ^{3}H-con A is a good measure for the con A binding sites available on the cell surface In contrast, the reaction with HRP and 3-3′-diaminobenzidin (DAB) plus H_2O_2 detects those binding sites that are occupied by con A in such a way that con A can still react with HRP This implies that the cytochemically detected con A (or con A-binding sites) constitute(s) a certain

Table 1 *Binding of ^{3}H-con A on confluent 3T3 cells after different treatments at room temperature*

Con A 2 ml con A for 15 min (50 μg/ml in PBS)
GA 2 % glutaraldehyde in 2 ml of 0 1 M cacodylate buffer, pH 7 2
PBS 2 ml of phosphate-buffered saline for 15 min
HRP 2 ml of horseradish peroxidase for 15 min (50 μg/ml in PBS)
DAB 2 ml of 3-3′-diaminobenzidin, activated with H_2O_2 for 10 min (500 μg/ml in 0 1 M Tris buffer with 0 9 % NaCl, pH 7 5)
OsO_4 1 % OsO_4 in 0 1 M cacodylate buffer, pH 7 2 for 1 h
α-MG 2 ml of 0 05 M α-methyl-D-glucoside in PBS for 15 min
Con A + α-MG 2 ml of a mixture of con A (50 μg/ml) and 0 05 M α-MG in PBS for 15 min

No	Stepwise treatments[a]	Non-prefixed		Prefixed[c]	
		Photograph (fig 1)	Cell-bound[b] ^{3}H-con A (dpm/10^6 cells)	Photograph (fig 1)	Cell-bound[b] ^{3}H-con A (dpm/10^6 cells)
1	Con A/GA/ /-/-/-	—	15 800	—	12 100
2	Con A/PBS/-/-/-/-	—	15 000	—	12 000
3	Con A/HRP/GA/DAB/OsO_4	A	15 400	B	12 200
4	Con A/GA/HRP/GA/DAB/OsO_4	C	15 800	like B	12 100
5	Con A/α-MG/HRP/GA/DAB/OsO_4	D	9 800	E	5 800
6	Con A + α-MG/HRP/GA/DAB/OsO_4	F	1 800	G	900

[a] All steps were separated by two washes in PBS
[b] DAB and OsO_4 were omitted when binding of ^{3}H-con A was measured
[c] Prefixation for 15 min in 2 5 % glutaraldehyde (GA) in 0 1 M cacodylate buffer

fraction of the total amount of cell-bound con A (or ^{3}H-con A detected con A binding sites)

The present study shows that the amount of cell-bound ^{3}H-con A cannot always be equated with the amount of cytochemically detected con A and that this discrepancy is probably caused by redistribution and concomitant change in accessibility of the con A binding sites on the plasma membrane

MATERIALS AND METHODS

BALB/c 3T3 mouse fibroblasts were grown on carbon-coated coverslips in plastic Petri dishes filled with 5 ml of Dulbecco's modified Eagle medium supplemented with 10 % newborn calf serum and antibiotics Petri dishes were seeded with $2\,5 \times 10^5$ cells and grown to confluence In con A binding experiments the dishes with attached confluent cells were rinsed in phosphate-buffered saline (PBS) and 2 ml of ^{3}H-con A in Ca^{2+} and Mg^{2+} containing PBS were added After exposure to ^{3}H-con A for 15 min at room temperature and other treatments as indicated in the legend to table 1, the cells were washed in PBS, scraped off the dishes, and their radioactivity was determined in a liquid scintillation counter The ^{3}H-con A had been made by New England Nuclear by exposing con A (Calbiochem) to 3 Ci of tritium gas for 2 weeks This material was purified in our laboratory by centrifugation and chromatography on Biogel 100 The purified ^{3}H-con A reacted the same as unlabeled con A in agglutination experiments and was indistinguishable in chromatographic behaviour

The cytochemical reaction of cell-bound con A with horseradish peroxidase and DAB plus H_2O_2 was carried out at room temperature on the cells in situ Essentially the method of Bretton et al [11] was used, with modifications as indicated in the legend to table 1 The mixture of con A and α-methyl-D-glucoside (α-MG) was made immediately prior to addition to the cells Cells were dehydrated in ethanol, embedded in a mixture of Epon and Araldite, separated from the glass, sectioned, and studied in the electron microscope without additional staining All electron micrographs were printed at a final magnification of 16 000

RESULTS

The results of the binding experiments of ^{3}H-con A with differently treated cells and of the cytochemical reaction of HRP-DAB after con A binding are summarized in table 1 and fig 1 When a mixture of ^{3}H-con A or

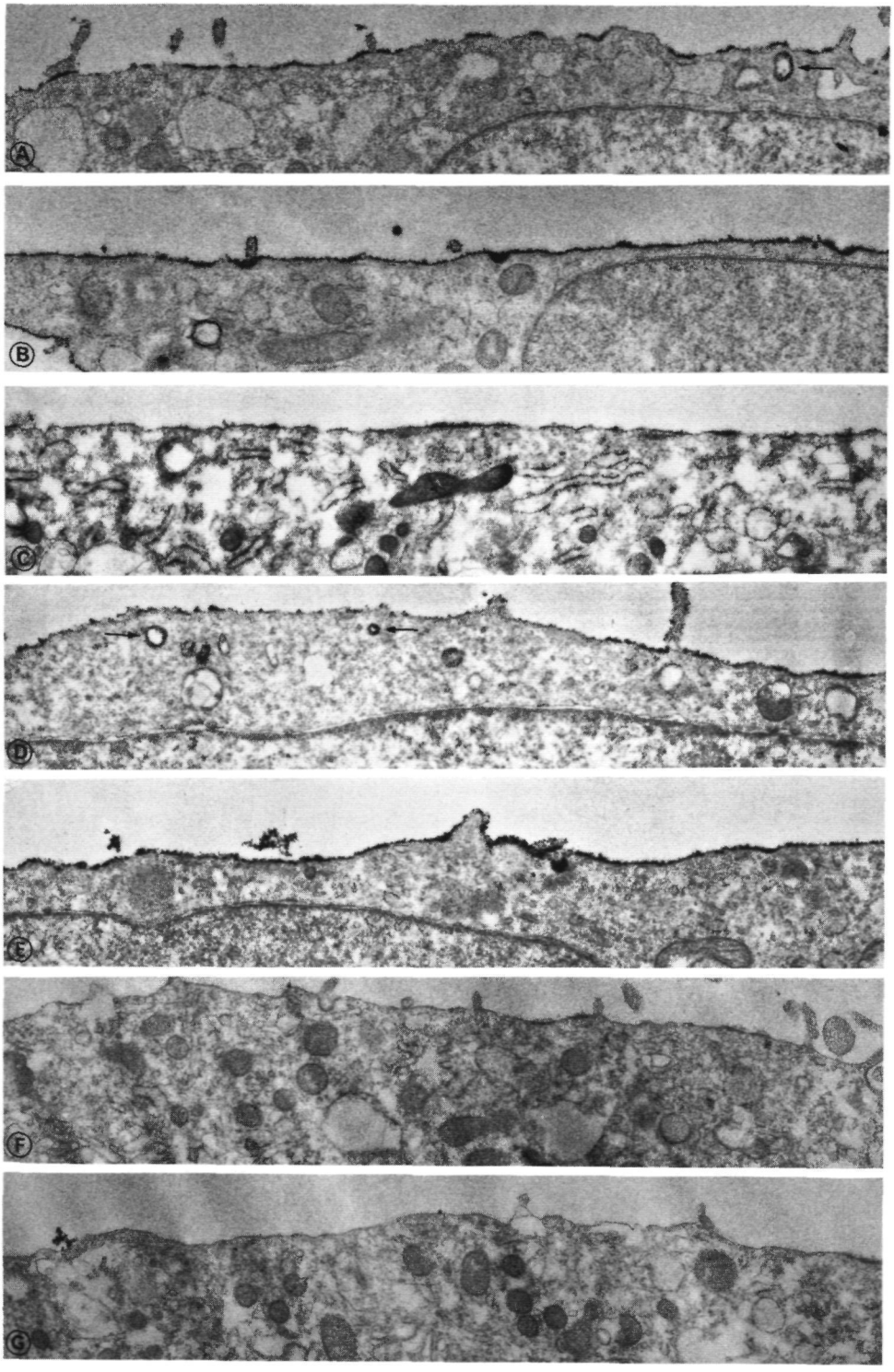

Fig. 1. 3T3 cells with conA specific HRP-DAB reaction product. For details, see table 1.

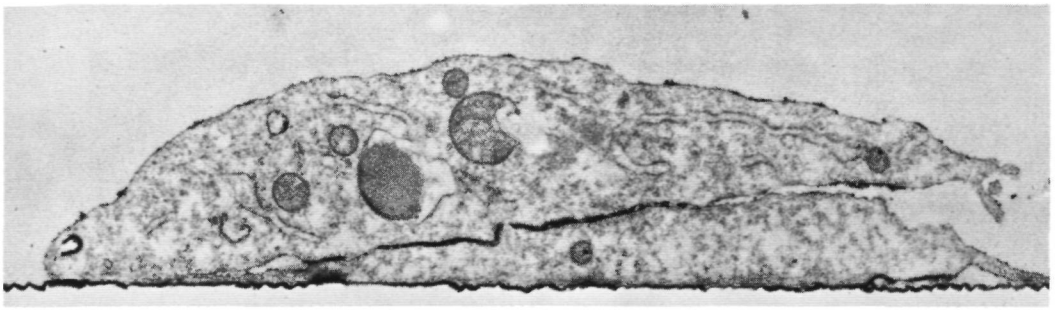

Fig. 2. Overlapping 3T3 cells with increased amount of HRP-DAB reaction product on adjacent plasma membranes. Treatment as in fig. 1 A.

conA and α-MG was added to non-prefixed or prefixed cells, practically no binding of ^{3}H-conA to the cells occurred and no cell-bound conA was detected in the electron microscope (table 1:6; fig. 1*F* and *G*). This indicated that the specific binding of ^{3}H-conA was not obscured by non-specific binding of ^{3}H-conA and that the cytochemical reaction of HRP-DAB with conA was not accompanied by unspecific attachment of autooxidized DAB [12].

Under different experimental conditions (table 1:1, 2, and 3) non-prefixed cells bound approx. 25% more ^{3}H-conA than prefixed cells. In non-prefixed cells part of the cell-bound conA was possibly taken up by endocytosis and new binding sites became available in this process. Non-prefixed cells showed a patchy distribution of the HRP-DAB reaction product that covered not more than 30–50% of the outer cell surface, whereas prefixed cells were completely covered with a similar layer of the same reaction product (fig. 1*A*, *B*). However, the amount of HRP-DAB reaction product on adjacent plasma membranes of partly overlapping non-prefixed cells had the same regular distribution as on the prefixed surface of non-overlapping cells (fig. 2).

The difference in amount of HRP-DAB reaction product between prefixed and non-prefixed cells was illustrated by the equal thickness of the electron-dense patches on the non-prefixed cells as compared with the electron-dense continuous layer on the prefixed cells. This difference was only partly due to endocytotic uptake (fig. 1*A*, *D*) of the cell-bound conA before HRP had been added. Binding experiments with α-MG had shown that approx. 80% of the cell-bound conA could be released by α-MG in 0.5 h. This means that not more than 20% of the conA had been taken up by endocytosis during that time. The greater amount of HRP-DAB reaction product after prefixation was due to an effect of glutaraldehyde on the membrane before conA addition and not on cell-bound conA, as was shown on cells that were prefixed after conA addition but before exposure to HRP (table 1:3 and 4; figs 1*A*, *B*, *C*). These cells had the same distribution of electron-dense reaction product as non-prefixed cells, indicating that the difference in distribution was caused by addition of conA to the non-prefixed membrane.

Incubation of non prefixed cells for 15 min with α-MG after addition of conA caused 30–50% release of cell-bound ^{3}H-conA (table 1:2, and 5). The electron micrographs of non-prefixed cells show that the amount of HRP-DAB reaction product decreased after incubation with α-MG (fig.

1 *A*, *D*) The amount of ^{3}H-con A bound to prefixed cells was reduced approx 50% by the same α-MG addition, but hardly less reaction product was attached to the cell membrane (fig 1 *B*, *E*) This confirms our conclusion that the amount of HRP-DAB reaction product is not indicative of the amount of cell-bound con A It suggests that other factors (e g membrane configuration, distribution of con A binding sites) are important in determining the amount of HRP-DAB reaction product

It should be noted that HRP does not remove the cell-bound con A from prefixed and non-prefixed cells (table 1 1, 2, and 3), although it binds con A with specific sugar groups in a similar way as α-MG Apparently the affinity of con A for the membrane sites is much greater than for HRP Thus the possibility can be excluded that the patch-like distribution of the HRP-DAB reaction product on non-prefixed cells (fig 1 *A*) is due to local removal of cell-bound con A by HRP

DISCUSSION

Our results show that under certain experimental conditions the amount of cell-bound con A is not reflected by the amount of cytochemically detected con A This discrepancy between binding and cytochemical detection is not due to lack of specificity of the HRP-DAB reaction, but seems to be related to the nature or distribution of the con A binding sites on the plasma membrane Immobilization of the cell membrane by chemical fixation or immobilization by partial overlapping of confluent 3T3 cells [13] results in more cell-bound con A detected by HRP-DAB Apparently con A binding sites on immobilized membranes are exposed in such a way that most membrane-bound con A molecules are coupled with HRP, thus facilitating an optimal reaction with DAB

Non-prefixed cells have a different distribution of the HRP-DAB reaction product It is very likely that the patch-like distribution of the electron-dense reaction product reflects the clustering effect [14] of the tetravalent con A [15, 16] on the membrane binding sites According to this theory con A induces movement of the sites to which it binds [17], resulting in a mosaic of local accumulations of cross-linked con A binding sites and areas free of these binding sites Thus the patches in our experiments would correspond to the clusters of cell-bound con A and the areas that are without reaction product would represent those parts of the membrane from which the con A-binding sites had been removed Similar results were reported recently by Nicolson [18], Inbar & Sachs [19], and Inbar et al [20], who worked with FITC-labeled con A on prefixed and non-prefixed 3T3 and lymphoma cells

Why the concentration of cell-bound con A in clusters is not reflected in our experiments by a greater amount of HRP-DAB reaction product in those areas, remains a difficult problem It may be due to the fact that con A is attached with all four active sites to membrane binding sites and cannot bind HRP [5], or the cell-bound con A molecules may be so close together that binding of HRP is impossible for steric reasons

Ferritin-conjugated con A (Fer-con A) has been used by others [17, 20, 21] to demonstrate the redistribution of con A binding sites on the plasma membrane Preliminary experiments in our laboratory indicate, however, that results obtained with Fer-con A may be complicated by redistribution as a result of an induced increase of endocytosis of the big Fer-con A complex by the cell

The results of our experiments on binding and cytochemical detection of cell-bound con A indicate that redistribution of con A binding sites can be demonstrated non-

quantitatively with HRP This redistribution depends on the mobility of the membrane because prefixation with glutaraldehyde as well as local immobilization in partly overlapping cells prevents the formation of patches

Redistribution of conA sites has been demonstrated with HRP in normal and transformed cells [10, 22–25] These studies showed that the redistribution on normal cells differs from that on transformed cells and suggested that this difference is responsible for the higher agglutinability of transformed cells as compared to normal cells. It should be kept in mind, however, that the detection of conA binding sites with HRP is non-quantitative and that only differences in the distribution of the patches can be demonstrated Differences in the number of conA binding sites within the patches escape detection by HRP It remains to be established whether the concentration of conA sites in the patches plays a role in agglutinability or not

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REFERENCES

1 Inbar, M & Sachs, L, Proc natl acad sci US 63 (1969) 1418
2 Inbar, M, Ben-Bassat, H & Sachs, L, Nature new biol 236 (1972) 3
3 Burger, M M, Nature 227 (1970) 170
4 — Growth control in cell cultures (ed G E Wolstenholme) Churchill-Livingstone, London (1971)
5 Bernhard, W & Avrameas, S, Exptl cell res 64 (1971) 232
6 Nicolson, G L & Singer, S J, Proc natl acad sci US 68 (1971) 942
7 Smith, S B & Revel, J-P, Dev biol 27 (1972) 434
8 Ozanne, B & Sambrook, J, Nature new biol 232 (1971) 156
9 Arndt-Jovin, D J & Berg, P, J virol 8 (1971) 716
10 Temmink, J H M & Collard, J G, Electron microscopy and cytochemistry, Proc 2nd int symp (ed E Wisse, W Th Daems, I Molenaar & P van Duijn) Drienerlo, The Netherlands (1973) p 299 North-Holland, Amsterdam (1974)
cancer 10 (1972) 397
11 Bretton, R, Wicker, R & Bernhard, W Int j cancer 10 (1972) 397
12 Hirai, K, J histochem cytochem 19 (1971) 434
13 Martz, E & Steinberg, M S, J cell physiol 81 (1973) 25
14 Nicolson, G L, Nature new biol 233 (1971) 244
15 Edelman, G M, Cunningham, B A, Reeke, G N, Becker, J W, Waxdal, M J & Wang, J L, Proc natl acad sci US 69 (1972) 2580
16 Sharon, N & Lis, H, Science 177 (1972) 949
17 de Petris, S & Raff, M C, Nature new biol 244 (1973) 275
18 Nicolson, G L, Nature new biol 243 (1973) 218
19 Inbar, M & Sachs, L, FEBS lett 32 (1973) 124
20 Inbar, M, Huet, Ch, Oseroff, A R, Ben-Bassat, H & Sachs, L, Biochim biophys acta 311 (1973) 594
21 Nicolson, G L & Singer, S J, Proc natl acad sci US 68 (1971) 942
22 Singer, S J & Nicolson, G L, Science 175 (1972) 720
23 Martinez-Palomo, A, Wicker, R & Bernhard, W, Int j cancer 9 (1972) 676
24 Rowlatt, C, Wicker, R & Bernhard, W, Int j cancer 11 (1973) 314
25 Huet, Ch & Herzberg, M, J ultrastruct res 42 (1973) 186

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A COMPARATIVE STUDY OF FOUR CYTOCHEMICAL DETECTION METHODS OF CONCANAVALIN A BINDING SITES ON THE CELL MEMBRANE

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SUMMARY

Four different electron cytochemical methods to detect concanavalin A (ConA) binding sites on the plasma membrane of mouse fibroblasts were compared in this study. The ConA binding sites were made visible either by adding ConA, followed by horseradish peroxidase (HRP) or hemocyanin (HC), or by marking the sites with complexes of ConA with ferritin (Fer) or with microperoxidase (MP). HC and Fer are directly visible in the electron microscope; HRP and MP are detected by their electron-dense reaction product with diaminobenzidin and H_2O_2. Differences in sensitivity of the ConA binding sites for the different markers were found and resulted in a tentative interpretation of the labelling reactions. All experiments suggested that normal and transformed murine fibroblasts both have plasma membranes in which the binding sites can move equally well and can be induced to form clusters. These results are discussed in relation with the hypothesis that differences in clustering of ConA sites between normal and transformed cells are responsible for differences in the agglutinability by ConA of these cells.

Concanavalin A (ConA) is probably the most widely used plant lectin in comparative studies of normal and transformed cells. ConA agglutinates transformed cells more easily than non-mitotic normal cells [1–5], and it can be made visible in the electron microscope in various ways. ConA is often used in experiments where a causal relation is sought between agglutinability and differences in growth control [6–8]. However, it is still uncertain how agglutination by ConA is correlated with number [1, 9–14] and distribution [15, 16] of ConA binding sites on the membrane.

In a previous report on normal and transformed murine fibroblasts we showed that increased agglutinability by ConA is not always correlated with changes in detectability of cell-bound ConA by horseradish peroxidase (HRP) and diaminobenzidin (DAB) [14]. In addition we showed that the cytochemical detection of cell-bound ConA with HRP–DAB is rather unquantitative, and that local differences in the amount of cell-bound ConA are not always reflected by local differences in the reaction product [17, 18]. In view of these findings it became important to investigate whether other detection methods of cell-bound ConA were more reliable in these respects.

In this investigation four electron cytochemical ConA detection methods are compared that differ either in the type of bond between ConA and the cytochemical marker, or in the size of the cytochemical marker or its reaction product. Ferritin (Fer) [19] and

microperoxidase (MP) [20, 21] were bound covalently to ConA before addition to the cells; HRP [22] and hemocyanin (HC) [23] were attached to cell-bound ConA by means of their sugar residues. Fer and HC are relatively big molecules with diameters of approx. 120 Å and 350 Å respectively, that are individually visible in the electron microscope; HRP and MP are only approx. 50 Å and 15 Å respectively, but their reaction product is diffuse and electron-dense.

A number of electron cytochemical studies of the cell membrane of fibroblasts have been published recently by other investigators, using one or more of the ConA detection methods employed in this study. Although the results of some of these investigations agree with the results described here [23, 24], many other papers have led to apparently conflicting results [25–33]. This may be largely due to the fact that different biological material was used for the different investigations. It has been suggested before that results on one type of cells cannot be considered valid for other cells [25]. We agree with that suggestion, but feel that all experimental data are relevant for the validity of more general hypotheses. The results of this comparative study will, therefore, be discussed in relation with the hypothesis that clustering of ConA binding sites [16, 28] induces agglutination of cells by ConA and that differences in agglutinability between normal and transformed cells are caused by differences in fluidity of the cell membranes [34, 35].

MATERIALS AND METHODS

Cell material and experimental conditions

The cells used in this study were normal 3T3 mouse fibroblasts (3T3) and Simian virus 40-transformed 3T3 cells (SV3T3). They differ markedly in saturation density when grown under the same conditions (3T3: 70000 cells/cm² SV3T3: 500000 cells/cm²), they have a very different agglutinability with ConA (midpoint agglutination of 3T3: 1 000 μg/ml; mpa of SV3T3, 15 μg/ml) [36], and in confluent cultures of 3T3 no S and G2 cells can be detected by impulse cytophotometry [37].

All cells were grown on carbon-coated coverslips in 5 cm plastic Petri dishes filled with 5 ml of Dulbecco's modified Eagle medium, supplemented with 10% newborn calf serum and antibiotics. The Petri dishes were seeded with 2.5×10^5 cells and grown to confluence in a humidified CO_2-incubator at 37°C. All treatments were carried out at room temperature. Only in experiments on the effect of low temperature (0°C) on the detectability and distribution of ConA binding sites were cells kept at 0°C for 0.5 h before treatment and all reactions were executed at 0°C with precooled reagents The effect of prefixation or trypsin treatment on the ConA binding sites was determined in experiments where the cells were treated with 2.5% glutaraldehyde (GA) for 15 min, or with 10^{-3}% trypsin for 5 min followed by two washes with egg-white trypsin inhibitor, before addition of the conjugated or free ConA. All reactions on the cells were carried out in situ. Cells were washed before and between treatments with Ca^{2+}- and Mg^{2+}-containing phosphate-buffered saline (PBS), unless otherwise indicated.

Cytochemical markers of ConA

For the electron microscopic detection of ConA binding sites by means of HRP–DAB and HC, 2 ml of ConA (50 μg/ml) (Calbiochem) were added to each Petri dish with cells. After 15 min exposure to ConA at room temperature or 30 min at 0 C, the reaction with HRP–DAB was done as described before [17]. The binding of HC to cell-bound ConA was carried out by adding 2 ml of a suspension containing 500 μg/ml HC to the cells for 15 min at room temperature or 30 min at 0°C [23]. The hemocyanin (prepared from *Helix pomatia*) was a generous gift from Dr E. F. J. van Bruggen, Laboratory of Structural Chemistry, University of Groningen, Groningen, The Netherlands. In control experiments 2 ml of a 1: 1 mixture of ConA (100 μg/ml) and 0 2 M α-methyl-D-glucoside (α-MG) were added to the cells, followed by the normal addition of HRP and DAB or HC.

In experiments with the ConA–Fer conjugate 2 ml of a ConA–Fer suspension containing 3 mg/ml Fer were added to the cells in a Petri dish for 15 min at room temperature or for 30 min at 0°C. To control experiments a similar ConA–Fer suspension, but with 0.1 M α-MG was added. The ConA–Fer conjugate was made by adding 1 ml of Fer (100 mg/ml) to 0.25 ml of PBS containing 10 mg ConA and 25 mg α-MG for ligand protection, and reacting the mixture with 0.04 ml of 2.5% GA for 1 h at 20°C. The reaction was stopped by adding 5 mg glycine to the mixture. After dialysis against 2×2 l of PBS for 24 h, the approximate concentration of ConA–Fer was determined by spectrophotometry (E_{280}^{Fer} (1 mg/ml; 1 cm) = 11.4).

For the reaction with ConA–MP, 2 ml of a suspension containing 100 μg/ml ConA–MP were left with the cells in a Petri dish for 15 min at room temperature or for 30 min at 0°C. After rinsing with

PBS and fixation with GA the reaction with DAB and H_2O_2 was done as in the case of HRP labelling, except that the reaction time with DAB was extended to 4 h because of the low activity per molecule of MP (see below) In control experiments 0 1 M α-MG had been mixed with the ConA–MP complex 15 min before addition to the cells

ConA–MP conjugate was made by adding 1 mg of MP (Sigma) in 0 2 ml of PBS and 0 1 ml of 1 % GA to 1 ml of ConA (10 mg/ml in PBS) that had been adsorbed to 2 ml of a thick slurry of Sephadex G-200 beads during 30 min The coupling reaction was stopped after 60 min at 20°C by adding 0 5 ml of glycine (40 mg/ml in PBS) for 30 min and the liquids were removed by sucking through a glass filter After a quick rinse with 2 ml glycine the ConA–MP was removed from the Sephadex beads by twice adding 3 ml of 0 1 M α-MG plus glycine (5 mg/ml) within 30 min Dialysis for 4 h against 1 l of glycine in PBS (5 g/l) and overnight against 2 l of 1 M NaCl in PBS resulted in a suspension with a precipitate The precipitate was removed by centrifugation (3 500 g, 15 min) and the remaining suspension was stable for at least 1 month when stored at 4°C Before use the NaCl was removed by dialysis against PBS The MP concentration was determined by spectrophotometry ($E^{MP}_{4[0}$ (1 , 1 cm) 430) and the ConA concentration likewise (E^{C}_{280} (1 %, 1 cm) 12) with correction for the MP adsorption (E^{MP}_{280} (1 %, 1 cm) 40) The molecular ratio of MP to ConA in the complex was calculated to be approx 2 5 By measuring the peroxidase activity on DAB + H_2O_2 in the spectrophotometer at 342 nm [38], the activity of the ConA–MP complex per mg MP was found to be 25 % of the HRP activity Because of the small size of MP this means that the activity per molecule MP is only approx 1 % of the HRP activity per molecule

Electron microscopy

The procedure for fixation, dehydration, and embedding for electron microscopy has been described before [17] No post section staining was applied to material treated with ConA HRP, ConA–Fer, or ConA MP Cells treated with ConA HC were stained on the grids with 2 5 % uranyl acetate in 50 % ethanol Photographs were taken in a Philips 300 electron microscope operating at 60 kV

All experiments were done at least twice and sections were made of each treatment at three different areas in the embedded material This resulted in at least twenty cross sections of cells per treatment Comparisons between differently treated cells were made only within the same experiment, where the amount of label added and the reaction times were exactly the same

RESULTS

Horseradish peroxidase

When normal 3T3 cells were treated with ConA and HRP–DAB, a discontinuous layer of reaction product was found on the outside of the cell membrane (fig 1 *A*) [14], whereas a continuous layer was found after prefixation with GA (fig 1 *B*) Because prefixed 3T3 cells do not bind more ConA than non-prefixed cells, this indicated that the HRP–DAB reaction is not always quantitative [17] The possible reasons will be discussed later Addition of HRP–DAB to non-prefixed SV3T3 cells resulted in a layer of reaction product that was more continuous than that of non-prefixed 3T3 cells, but not completely continuous as in prefixed 3T3 and SV3T3 cells (fig 1 *C*) Trypsin treatment did not influence the amount and distribution of the DAB reaction product on the surface of the 3T3 cells (fig 1 *D*) [14] However, reacting 3T3 cells with HRP and DAB at 0°C resulted in a layer of reaction product that was less electron-dense than on 3T3 cells treated at room temperature, but with the same distribution (fig 1 *E*)

Cross sections of the cell surface show only relatively small areas of the membrane Therefore we studied sections parallel to the cell layer through the upper cell membrane, making a somewhat larger area of the cell membrane visible Parallel sections through 3T3 (fig 2 *A*) and SV3T3 (fig 2 *B*) showed the same difference in distribution of HRP–DAB reaction product as cross sections

Hemocyanin

In order to investigate whether the results with HRP–DAB labelling of cell-bound ConA had been influenced by the nature of the cytochemical reaction on the cell surface, the ConA on prefixed and non-prefixed 3T3 and SV3T3 cells was made visible with HC If the size of the marker molecule is relevant, the results of these experiments could be expected to differ from the previous ones If, however, the type of bond between ConA

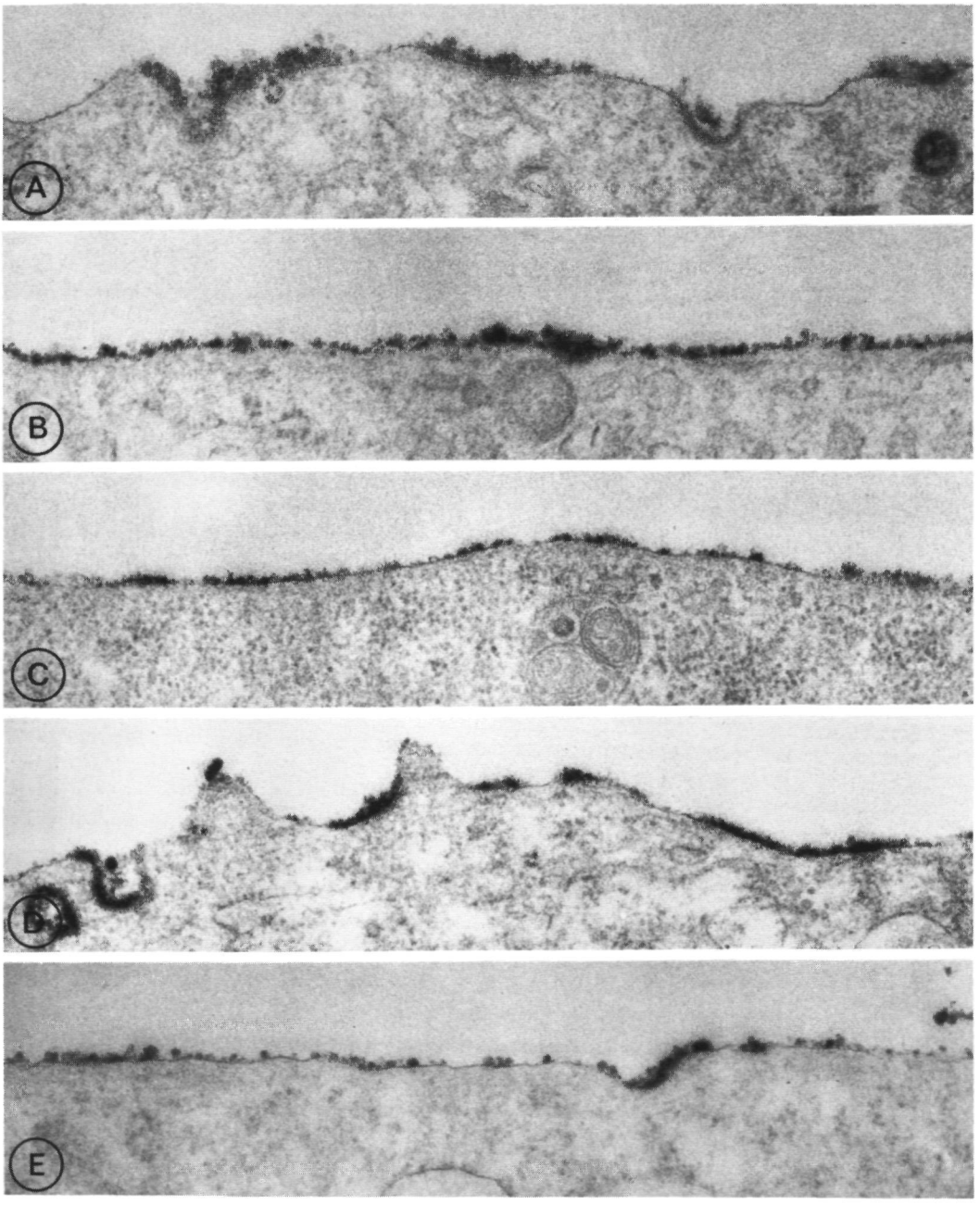

Fig. 1. Mouse fibroblasts treated with ConA and HRP–DAB. (*A*) 3T3, non-prefixed; (*B*) 3T3, prefixed; (*C*) SV3T3, non-prefixed; (*D*) 3T3, trypsin-treated; (*E*) 3T3, treated at 0°C. × 50 000.

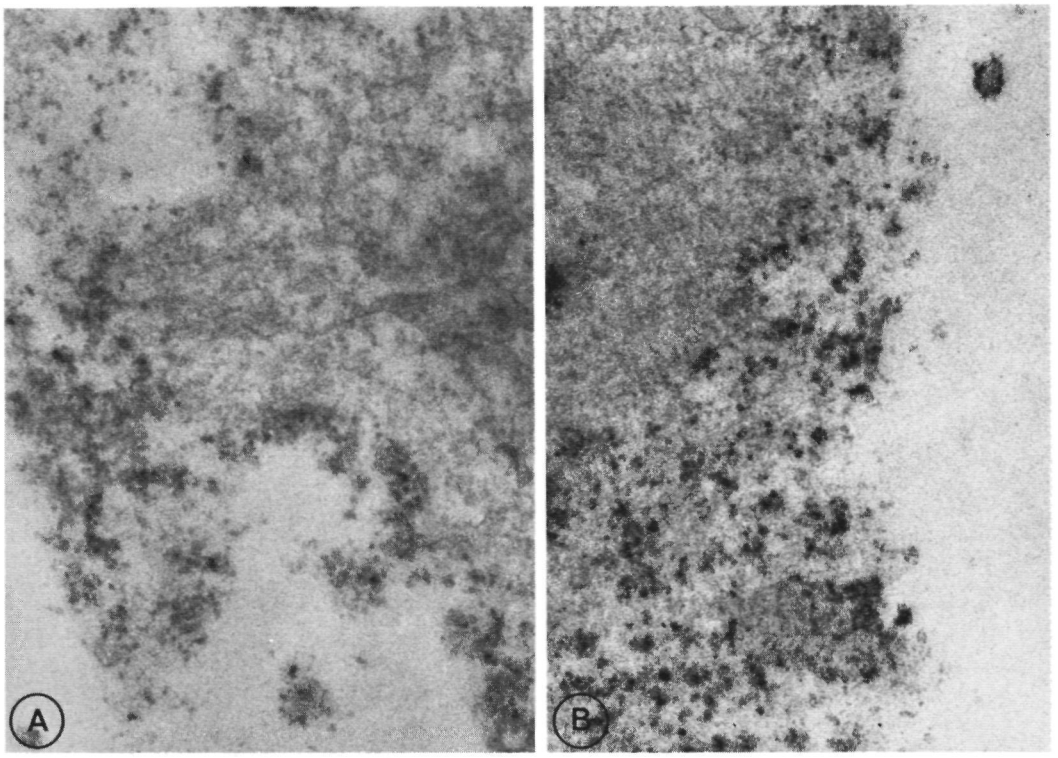

Fig. 2. Parallel sections of mouse fibroblasts treated with ConA and HRP–DAB. (*A*) 3T3, non-prefixed; (*B*) SV3T3, non-prefixed. × 50 000.

and the cytochemical marker is of prime importance, labelling of cell-bound ConA with HRP–DAB or with HC should give comparable results.

Non-prefixed 3T3 cells treated with ConA and HC had relatively few detectable HC molecules on the cell surface. The distribution tended to be discontinuous, with large areas completely free of marker molecules, intercalated by small areas with clusters of HC molecules (fig. 3*A*). Non-prefixed SV3T3 cells had a similar distribution of HC molecules, but their number was generally higher than in 3T3 cells (fig. 3*B*) and sometimes the discontinuity was less pronounced. In 3T3 as well as SV3T3 cells the number of HC molecules could not be increased by adding higher concentrations of ConA to the cells, or by extending the incubation times. Completed endocytotic uptake of HC could be observed in a few instances (fig. 4*B*), but relatively many HC molecules were often found in areas where endocytosis seemed to begin (fig. 4*C*, *D*). Prefixed 3T3 and SV3T3 cells treated with ConA and HC had 2–5 times more HC molecules attached to the cell membrane than non-prefixed cells and the distribution of these molecules was less irregular (fig. 3*C*, *D*). In order to decrease active endocytosis or rearrangement of ConA binding sites, non-prefixed 3T3 and SV3T3 cells were also treated with ConA and HC at 0°C. The number of HC molecules attached to these cells was extremely small (fig. 3*E*; not shown for SV3T3). This may result from increased rigidity of the membrane and its

binding sites at the low temperature It might also be due to the low reaction speed between ConA and its specific sites on the membrane and between cell-bound ConA and HC, or it might be due to the fact that ConA is a dimer at 0°C [39] In prefixed cells treated at 0°C the number of attached HC molecules was similarly small (fig 3*F*, not shown for SV3T3) Treatment of 3T3 cells with trypsin did not increase the number of HC-labelled ConA molecules on the cell membrane (fig 4*A*)

In all experiments HC molecules were found attached to the carbon-coated glass on which the cells were grown (fig 4*E*) and to extracellular material (fig 4*F*) This reaction was due to binding of ConA to the glass and the extracellular material It did not result from aspecific binding of HC, because it could be prevented in control experiments were α-MG had been added to ConA (fig 4*G*)

The distribution of HC-labelled ConA on normal and transformed 3T3 cells was also studied on replicas The results have been published elsewhere [18] and agree with the data presented here and with those of Smith & Revel [23]

When experiments with HRP–DAB as ConA label were compared with those where HC was used as marker for ConA, the information obtained in both cases on the distribution of ConA binding sites on the surface of 3T3 and SV3T3 cells was similar in many respects This suggested that the secondary binding of the marker to cell-bound ConA had influenced out experimental data greatly

Ferritin

In order to determine the effect of the bond between ConA and the marker molecule on the detection of ConA binding sites, we did experiments where the marker was linked to the ConA molecule in a different manner. Fer and MP covalently bound to ConA were used for that purpose, because they can be detected in the electron microscope in the same way as HC and HRP, Fer molecules can be seen individually, MP molecules can be traced by their DAB reaction product

The cell membrane of non-prefixed 3T3 cells treated with Fer–ConA was covered with the marker molecules (fig 5*A*) The distribution on the surface was always somewhat irregular, leaving small areas free of the conjugate Fer–ConA was often detected in endocytotic vesicles and in invaginations of the cell membrane that seemed to be early stages of endocytosis When non-prefixed SV3T3 cells were treated with Fer–ConA, they invariably were covered with more label than the non-prefixed 3T3 cells that were treated with the same preparation (fig 5*B*) The distribution of the label over the cell surface was not more irregular than in 3T3 cells In contrast to the results obtained with HRP and HC, prefixed 3T3 and SV3T3 cells treated with Fer–ConA has less marker molecules on their surface than their non-prefixed, similarly treated counterparts (fig 5*C*, *D*) (see however [24]) This difference in amount of cell-bound Fer–ConA between prefixed and non-prefixed cells was not accompanied by a difference in distribution over the cell surface Although treatment of the cells at 0°C decreased endocytosis of cell-bound Fer–ConA, the total amount of Fer ConA on the cell surface was always less than on comparable cells treated at room temperature (fig 5*E*, *F*) Trypsin seemed to increase the endocytosis of cells, but had no effect on the distribution or the total amount of Fer–ConA bound by 3T3 cells (fig 6*A*) Some binding of label to the glass was found (fig 6*B*), but this could be prevented by α-MG, like the binding of Fer–ConA to the

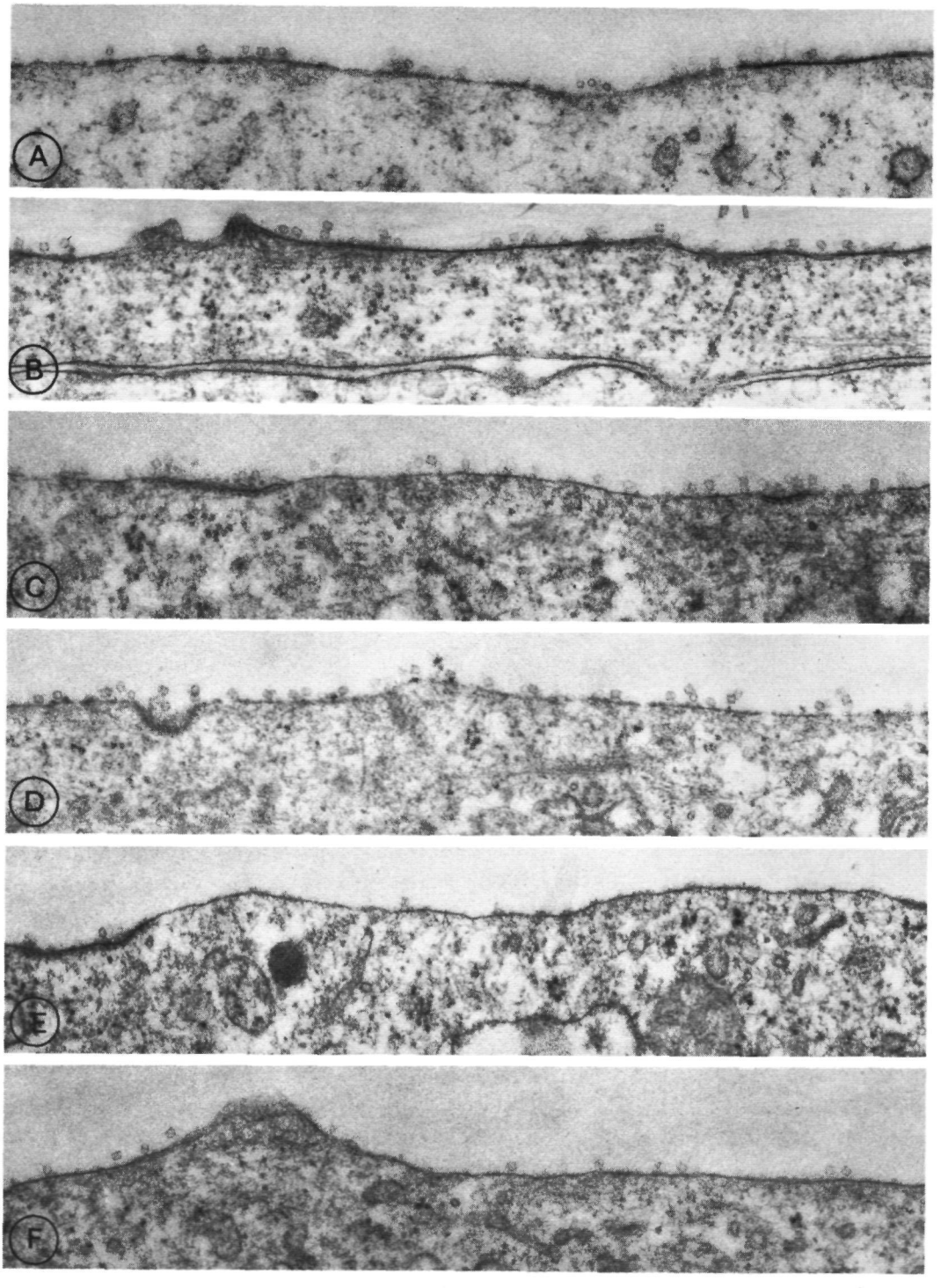

Fig. 3. Mouse fibroblasts treated with ConA and HC. (*A*) 3T3, nonprefixed; (*B*) SV3T3, non-prefixed; (*C*) 3T3, prefixed; (*D*) SV3T3, prefixed; (*E*) 3T3, treated at 0°C; (*F*) 3T3, prefixed and treated at 0°C. × 50 000.

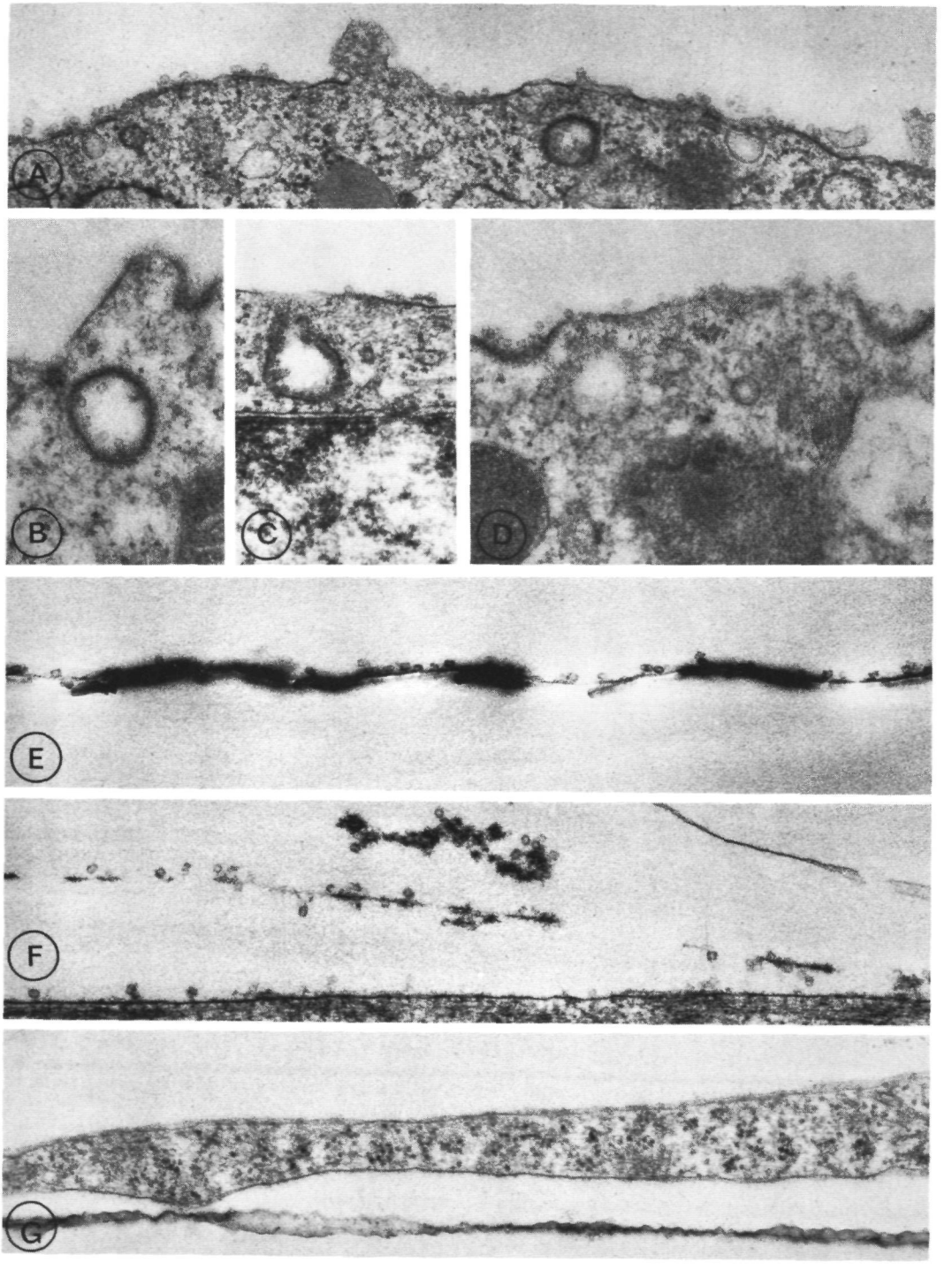

Fig. 4. Mouse fibroblasts treated with ConA and HC. (*A*) 3T3, trypsin-treated; (*B*) endocytotic vesicle with HC molecules; (*C*) (*D*) invaginations of cell membrane with HC molecules; (*E*) HC-labelling of cell-free glass surface; (*F*) HC-labelling of extracellular material; (*G*) 3T3, treated with α-MG. × 50 000.

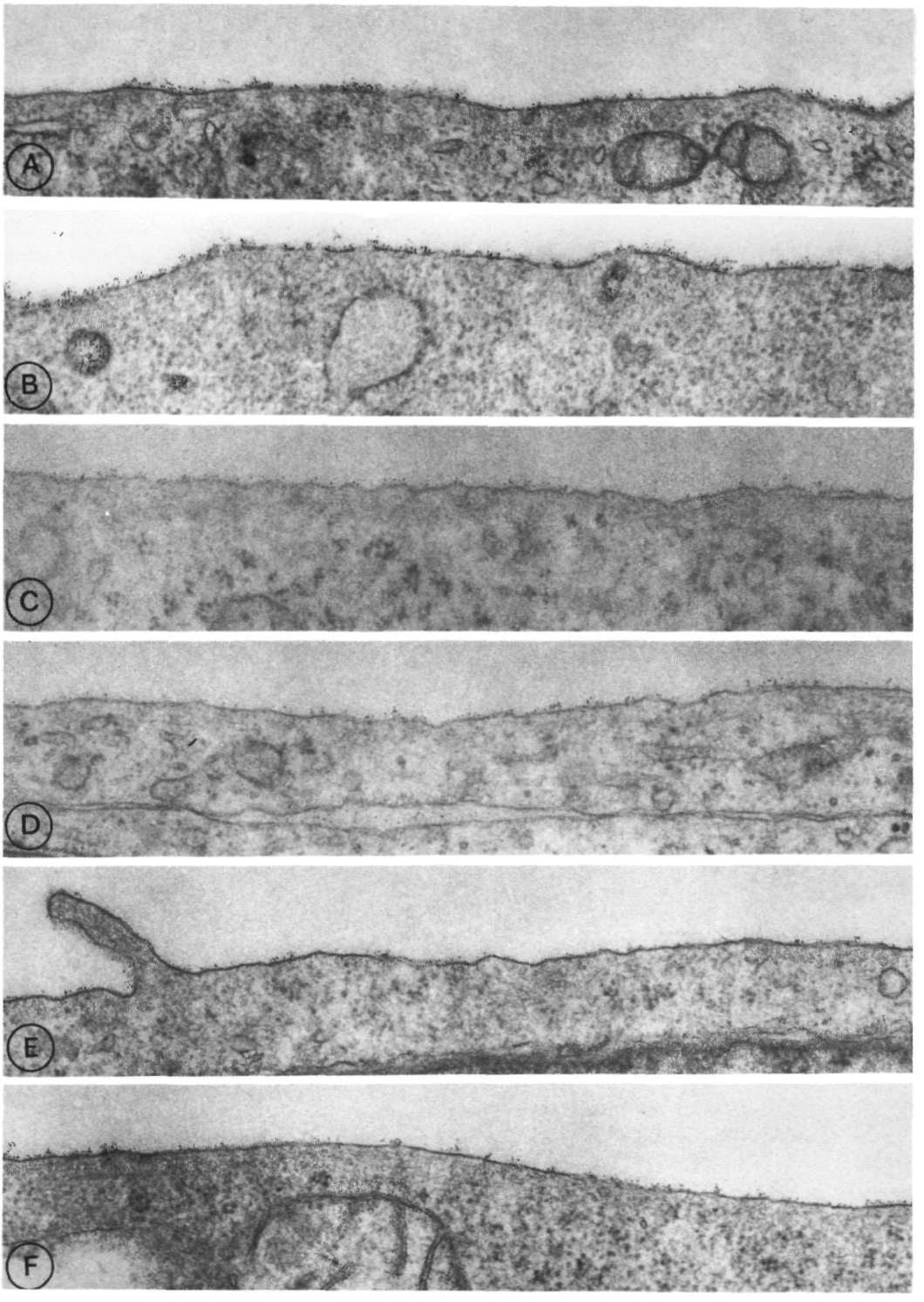

Fig. 5. Mouse fibroblasts treated with ConA–Fer. (*A*) 3T3, non-prefixed; (*B*) SV3T3, non-prefixed; (*C*) 3T3, prefixed; (*D*) SV3T3, prefixed; (*E*) 3T3, treated at 0°C; (*F*) SV3T3, treated at 0°C. × 50 000.

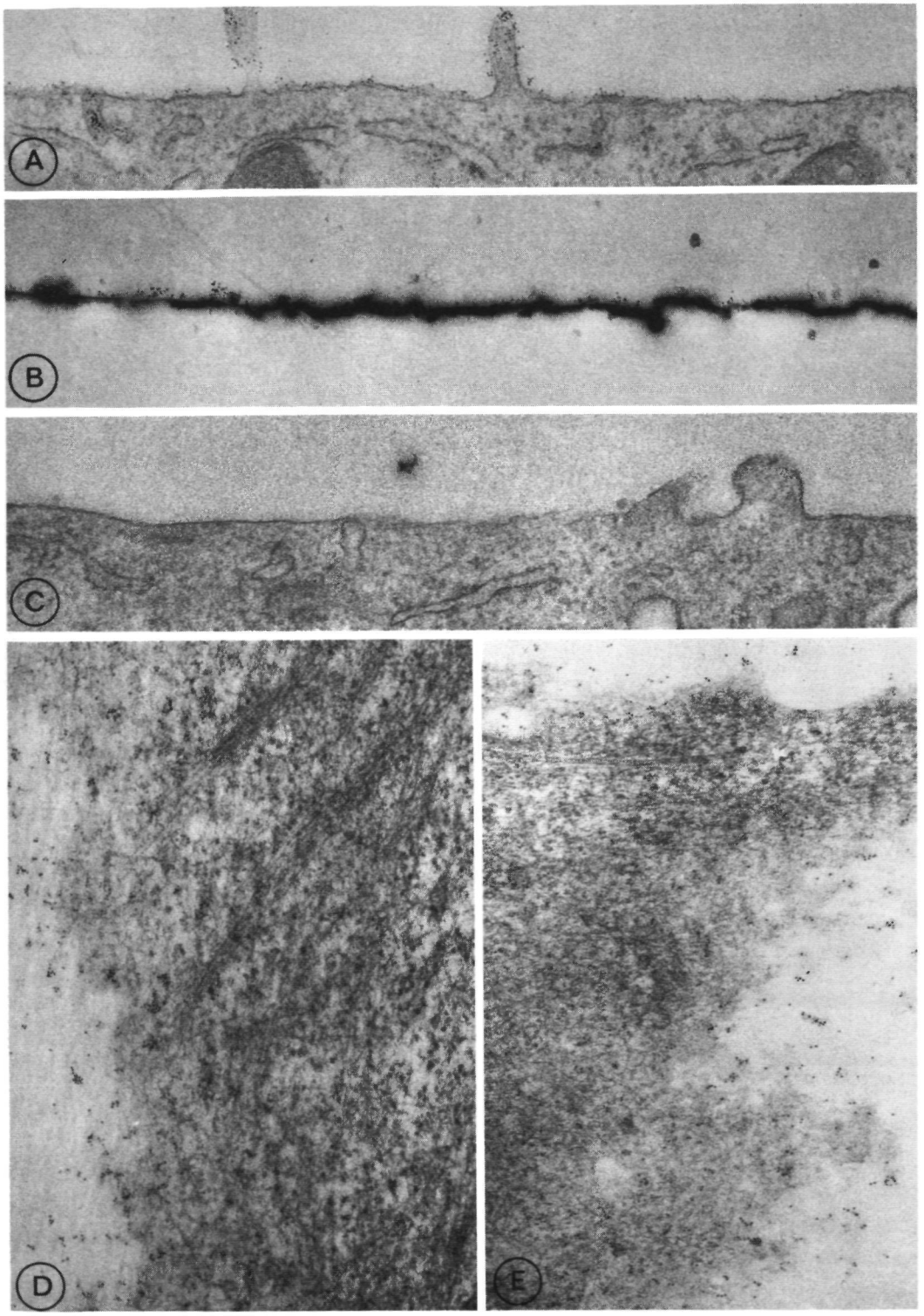

Fig. 6. Mouse fibroblasts treated with ConA–Fer (*A*) 3T3, trypsin-treated; (*B*) Fer-labelling of cell-free glass; (*C*) 3T3, treated with α-MG; (*D*) parallel section of 3T3, non-prefixed; (*E*) parallel section of SV3T3, non-prefixed. × 50 000.

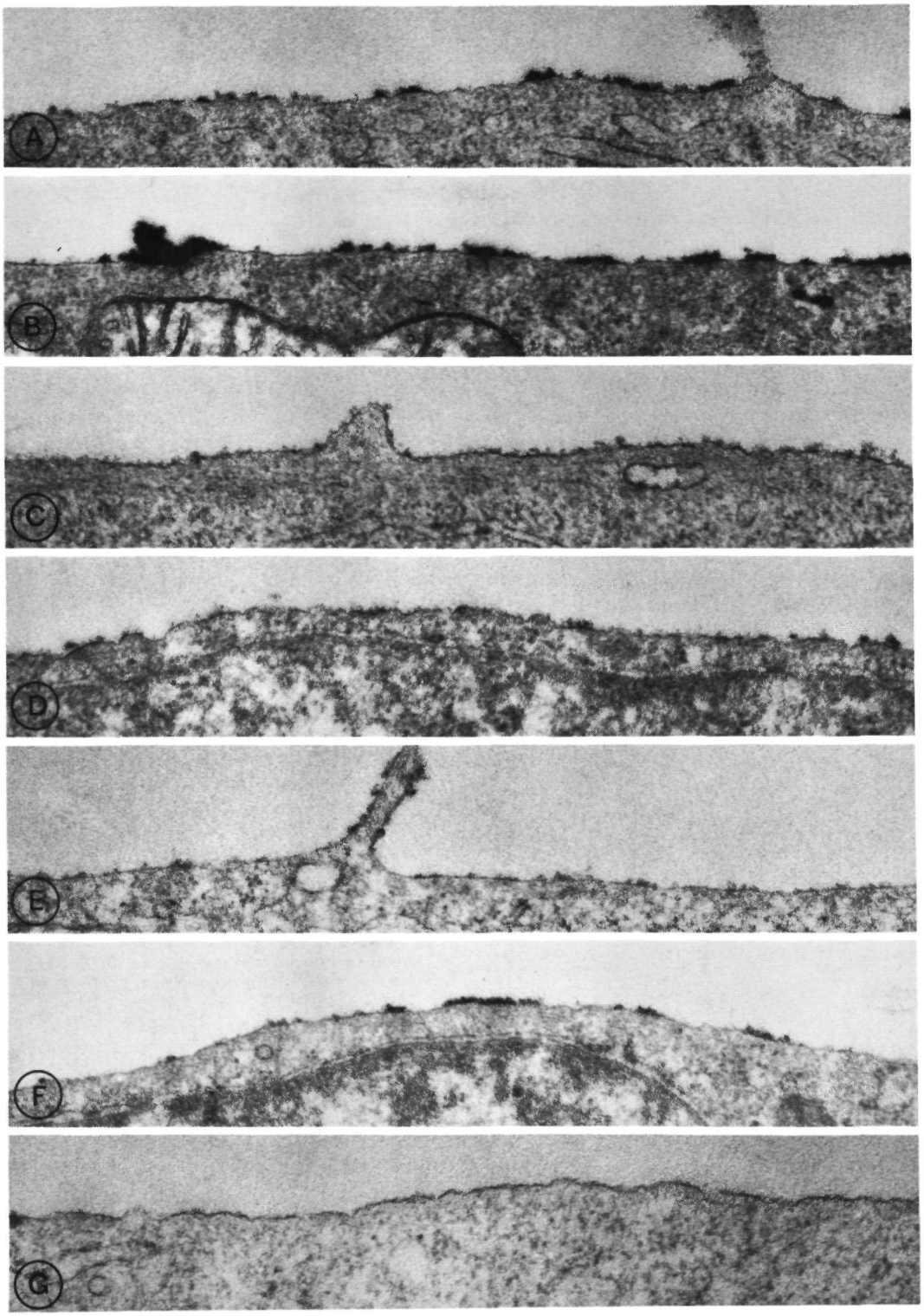

Fig. 7. Mouse fibroblasts treated with ConA–MP. (*A*) 3T3, non-prefixed; (*B*) SV3T3, non-prefixed; (*C*) 3T3, prefixed; (*D*) SV3T3, prefixed; (*E*) 3T3, treated at 0°C; (*F*) 3T3, trypsin-treated; (*G*) 3T3, treated with α-MG. × 50 000.

Table 1. *Estimated relative amount of cytochemically detected ConA after various treatments, as compared with agglutinability and amount of cell-bound ConA*

	3T3					SV3T3				
	Normal	Prefix	0°C	Tryp.	α-MG	Normal	Prefix	0°C	Tryp.	α-MG
Agglutination		−	−	− + +	−	− −	+ ±	− −		−
Binding	0.13	0.11	0.11	0.12	0.01	0.30	0.24	0.17		0.01
HRP	× × ×[a]	× × × × ×	× ×	× × ×	−	× × × ×	× × × × ×		× × ×	−
HC	× × ×[a]	× × × × ×	×	× × ×	−	× × × ×	× × × × ×	× ×	× ×	-
Fer	× × ×[a]	× ×	×	× ×	−	× × × ×	× ×	×	× ×	−
MP	× × ×[a]	×	×	× ×	−	× × × ×	× ×	×	× × ×	−

[a] The amount of reaction product on normal 3T3 cells is arbitrarily taken as standard (×) for the cytochemical marker used. The amount is estimated by the number of marker molecules (HC, Fer) or the length of cross-sectioned membrane covered with electron dense reaction product (HRP, MP).

Agglutination: Determined 20 min after addition of 25 μg/ml ConA as described previously [39].
Binding: Determined 20 min after adding 25 μg/ml ^{3}H-ConA (prepared as previously described [17]) to 1×10^6 cells suspended with 5×10^{-5} M EDTA, and expressed in μg/cm^2 on the assumption that the cells are perfect spheres.
HRP: ConA (50 μg/ml), 15 min; HRP (50 μg/ml), 15 min; DAB (500 μg/ml) H_2O_2, 10 min.
HC: ConA (50 μg/ml), 15 min; HC (500 μg/ml), 15 min.
Fer: ConA–Fer (approx. 3 mg/ml), 15 min.
MP: ConA–MP (100 μg/ml), 15 min; DAB (500 μg/ml) + H_2O_2, 10 min.

cells (fig. 6*C*). Fer-labelled ConA was also present on the microvilli (fig. 5*E*, 6*A*).

Parallel sections through unfixed cells labelled with Fer–ConA showed larger surface areas of the cells and confirmed that little difference in distribution of Fer–ConA existed between 3T3 (fig. 6*D*) and SV3T3 cells (fig. 6*E*).

Cross-linking of ConA and Fer with GA may make the ConA less reactive with its specific binding sites [46]. To exclude that not all ConA binding sites on the membrane were occupied with Fer–ConA for that reason, in some experiments double the amount of Fer–ConA was added to the cells. This did not significantly increase the number of Fer–ConA molecules on the cell membrane. Therefore we concluded that in all experiments enough Fer–ConA had been added to saturate all detectable ConA binding sites. The possibility that part of the binding sites remain unlabelled because they have a lower affinity for ConA or Fer–ConA seems excluded by the failure to detect classes of ConA binding sites with different affinities in time-dependent binding experiments with ^{3}H-ConA [14].

Microperoxidase

The experiments where MP–ConA had been added to the cells and exposed to DAB gave a different amount and distribution of the reaction product on the cells, than after HRP–DAB treatment. Non-prefixed SV3T3 cells had more surface membrane covered with reaction product than non-prefixed 3T3 cells (fig. 7*A*, *B*). Prefixed 3T3 and SV3T3 cells did not have this difference, but had distinctly less or less electron-dense DAB reaction product than non-prefixed cells (fig. 7*C*, *D*). The effect of trypsin and of exposing cells at 0°C was similar to the effect of these treatments to cells labelled with Fer–ConA (fig. 7*E*, *F*). The reaction of MP–ConA with the cells could be inhibited by mixing α-MG with the MP–ConA (fig. 7*G*).

Table 1 summarizes the results of this comparative study, and compares them with

the agglutinability and the total amount of cell-bound ConA, as determined in agglutination tests and binding experiments using ^{3}H-ConA Because of the different cytochemical markers and the experimental conditions, the reaction on non-prefixed 3T3 cells was arbitrarily chosen as the standard (× × ×) for each marker The amount of reaction product is expressed rather crudely and based on the number of particles per surface area (Fer and HC) or the length of membrane covered with DAB reaction product (HRP and MP) All detection methods of ConA binding sites were able to register the increased number of binding sites per unit surface area on SV3T3 cells as compared to 3T3 cells Prefixation of cells had a different effect on the detectability of ConA sites by markers attached to cell-bound ConA by sugar groups than by markers covalently bound to ConA Reacting the cells with ConA at 0°C resulted in less detectable lectin on the cell surface, even though endocytosis presumably does not occur at this temperature Trypsin treatment of 3T3 cells did not affect the amount of detectable ConA on the cell membrane greatly Increased endocytosis by trypsin treatment could be confirmed, but seemed unimprotant, relative to the endocytosis already occurring in our cells exposed to ConA and the marker molecules In all experiments non-specific reactions were seen The aspecificity concerned the reaction with ConA-specific sugar groups outside the cells and on the glass Control experiments with α-MG indicated that aspecific adsorption of ConA was not involved

DISCUSSION

Despite the greatly differing detection methods used in this study on number and distribution of ConA binding sites, some results are in remarkably good agreement with each other. All our experiments confirmed that SV3T3 cells have more ConA binding sites per unit surface area than 3T3 cells (table 1) [11, 13, 14, 15] Because of the smaller size of SV3T3 cells this agrees with the finding that the number of ConA sites per cell is the same for SV3T3 and 3T3 However, it has been suggested that a greater number of ConA binding sites per unit surface area is not sufficient cause for greater agglutinability [15, 40] We were able to confirm this by showing with all methods used in this study, that mild trypsin treatment of normal cells does not increase the number of detectable ConA binding sites or the amount of cell-bound ^{3}H-ConA, although trypsin treatment is known to increase the agglutinability of these cells (table 1) [1, 3, 11, 14, 21]

Another conclusion can be drawn from this investigation Non-prefixed SV3T3 and 3T3 cells both have a distinctly irregular distribution on the cell surface of detectable ConA binding sites This clustering phenomenon has been associated with mobility of ConA binding sites in the plane of the membrane [28, 34] According to this theory greater fluidity of the membrane would result in more pronounced clustering of ConA sites and this might explain greater agglutinability by ConA [15, 34] However, in all experiments on non-prefixed cells, we found that the irregularity of ConA distribution on 3T3 cells was at least equal to that on SV3T3 cells, whereas clear differences in agglutinability were detected In this respect, clustering of ConA sites on mouse fibroblasts clearly differs from more active processes like patching and capping on lymphocytes [41] Thus, our results seem to exclude the possibility that differences in agglutinability of 3T3 cells by ConA are caused by induced differences in distribution of ConA binding sites on the cell membrane Other surface

phenomena would seem to be responsible for differences in agglutinability

It might be reasoned that our 3T3 cells are not quite normal because their agglutination after 20 min with 25 μg/ml ConA was rated at + (table 1), whereas others found no agglutination whatsoever with 3T3 at the same ConA concentration [1, 3, 11, 15, 27] The discrepancy is probably merely due to differences in the degree of clumping deemed necessary to rate the agglutination at + Differences in growth pattern, saturation density and midpoint agglutination clearly show that our 3T3 cells should be considered normal and not spontaneously transformed in tissue culture

In many experiments ConA-specific label was detected in areas of the carbon-coated coverslip where no cells were growing We agree with previously made suggestions that this is probably due to ConA binding to sugar residues present in serum components from the growth medium [42, 43] It may well be that the binding of ConA to the cells is also partly mediated by serum components still adhering to the cell surface after several washes It should, however, be noted that this does not greatly influence the present theories on cell agglutination by ConA, but may only induce a modification of our notion of the cell membrane [44]

The application of four different ConA detection methods led to one important difference between the results. Prefixation of the ConA binding sites with GA increased the detectability of these sites by cytochemical markers bound indirectly via sugar residues to the cell-bound ConA (HRP, HC), whereas prefixation decreased the detectability of sites by markers that were directly bound covalently to ConA (Fer, MP), prior to addition to the cells A tentative explanation for this difference can be offered

In a previous report on the detection of ConA binding sites by HRP [17], we suggested that presence or absence of HRP–DAB reaction product on the cell membrane resulted from clustering induced by ConA and that the increased number of ConA sites within a cluster could not be detected for steric reasons The present results with other markers confirm these hypotheses and call for some addition Exposure of the specific sites on a cell membrane to the tetravalent ConA undoubtedly tends to increase the clustering of these sites by cross-linking This process may even be extended by adding markers with many ConA specific sugar residues, like HRP or HC The presence of relatively many Fer or HC molecules on some membrane areas, separated by areas that are practically free of label, clearly illustrate this difference in concentration of ConA binding sites resulting from clustering The high concentration of ConA binding sites within a cluster should, however, also be reflected by an increased density or extension of the HRP DAB and the MP DAB reaction product Exposure of non-prefixed cells to MP and DAB does indeed result in areas with a more electron-dense DAB reaction product than the reaction product that is evenly distributed over similarly treated prefixed cells (fig 7*A–D*) Apparently the HRP–DAB reaction is incapable of registering the same difference in concentration of ConA binding sites This is possibly due to two circumstances

(1) The molecular activity of HRP is so high at room temperature that even few cell-bound HRP molecules give a saturated patch of DAB reaction product, making the expression of additional HRP molecules in clusters on non-prefixed membranes impossible This is supported by the fact that a decrease in DAB reaction product is found only when few HRP molecules are reacted with DAB at 0°C

(2) In a cluster of non-prefixed ConA binding sites, the ConA molecules may be more saturated with membrane sugar residues and less able to bind HRP. This would be virtually impossible in cells where the sugar residues had been immobilized by prefixation. The second reason could also partially explain why the number of HC molecules bound to prefixed membranes with ConA is higher than on non-prefixed membranes. In the case of HC the enormous size of the HC molecule with its many sugar groups could be an additional reason for this difference in number of cell-bound HC molecules between prefixed and non-prefixed cells. Each HC molecule could easily be attached to several cell-bound ConA molecules, causing an underestimation of the number of cell-bound ConA molecules, especially in areas with high concentrations of ConA molecules, like clusters.

These considerations would seem to lead to the conclusion that ConA labelled covalently in vitro with cytochemical markers is a better detector of ConA binding sites than ConA labelled in situ with markers that form links via sugar residues. However, with the covalently bound cytochemical markers the detectability of ConA binding sites is also far from 100 % for several reasons. The covalent bond between ConA and the marker seems to make the complex less pliable and therefore less able to reach all binding sites. The effect can be expected to be greater in prefixed cells, where the binding sites themselves have also been made immobile [17]. This might account for the decreased number of detected ConA binding sites on prefixed membranes as compared to non-prefixed cells. In addition, the size of the marker molecule (e.g. Fer) may prohibit labelling of each ConA binding site [45], and the labelled ConA may be less reactive with its binding sites on the membrane due to the reaction with GA [46]. In view of these difficulties inherent in the use of electron cytochemical detection methods for ConA binding sites, the correspondence between the results obtained with the different labelling techniques is remarkable. It has been possible to confirm the existence of clustering of ConA binding sites in the membrane and to show that differences in clustering between cells are not a sufficient cause for differences in agglutinability of these cells.

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REFERENCES

1. Inbar, M & Sachs, L, Proc natl acad sci US 63 (1969) 1418.
2. Burger, M M, Proc natl acad sci US 62 (1969) 994.
3. Inbar, M & Sachs, L, Nature 233 (1969) 710.
4. Ben-Bassat, H, Inbar, M & Sachs, L, J membrane biol 6 (1971) 183
5. Inbar, M, Ben-Bassat, H & Sachs, L, Proc natl acad sci US 68 (1971) 2748.
6. Burger, M M, Nature 227 (1970) 170.
7. Burger, M M & Noonan, K D, Nature 228 (1970) 512.
8. Inbar, M, Ben-Bassat, H & Sachs, L, Nature new biol 236 (1972) 3.
9. Noonan, K D & Burger, M M, J biol chem 248 (1973) 4286.
10. — J cell biol 59 (1973) 134.
11. Ozanne, B & Sambrook, J, Nature new biol 232 (1971) 156.
12. Cline, M J & Livingston, D C, Nature new biol 232 (1971) 155.
13. Arndt-Jovin, D & Berg, P, J virol 8 (1971) 716.
14. Temmink, J H M & Collard J G, Electron microscopy and cytochemistry. Proc 2nd int symp (ed E Wisse, W Th Daems, I Molenaar & P van Duijn) Driehuis-Velsen, The Netherlands, 1973, p 299. North-Holland, Amsterdam (1974).
15. Nicolson, G L, Nature new biol 233 (1971) 244.
16. Loor, F, Exptl cell res 82 (1973) 415.
17. Collard, J G & Temmink, J H M, Exptl cell res 86 (1974) 81.
18. Collard, J G, Temmink, J H M & Smets, L A, Concanavalin A, Proc int symp (ed T K Chowdhury & A K Weiss) Norman, Oklahoma, 1974. Plenum, New York (1975).
19. Nicolson, G L & Singer, S J, Proc natl acad sci US 68 (1971) 942.
20. Feder, N, J histochem cytochem 18 (1970) 911.
21. Stobo, J D & Rosenthal, A S, Exptl cell res 70 (1972) 443.

22. Bernhard, W & Avrameas, S, Exptl cell res 64 (1971) 232.
23 Smith, S B & Revel, J-P, Dev biol 27 (1972) 434.
24. Petris, S de, Raff, M C & Mallucci, L, Nature new biol 244 (1973) 275.
25 Garrido, J, Burglen, M-J, Samolyk, D, Wicker, R & Bernhard, W, Cancer res 34 (1974) 230.
26. Ukena, T E, Borysenko, J Z, Karnovsky, M J & Berlin, R D, J cell biol 61 (1974) 70.
27. Rosenblith, J Z, Ukena, T E, Yin, H H, Berlin, R D & Karnovsky, M J, Proc natl acad sci US 70 (1973) 1625.
28. Nicolson, G L, Nature new biol 239 (1972) 193.
29. Huet, Ch & Bernhard, W, Int j cancer 13 (1974) 227.
30. Rowlatt, C, Wicker, R & Bernhard, W, Int j cancer 11 (1973) 314
31. Martinez-Palomo, A, Wicker, R & Bernhard, W, Int j cancer 9 (1972) 676.
32. Bretton, R, Wicker, R & Bernhard, W, Int j cancer 10 (1972) 397
33. Comoglio, P M & Guglielmone, R, FEBS lett 27 (1972) 256
34. Singer, S J & Nicolson, G L, Science 175 (1972) 720
35. Nicolson, G L, Nature new biol 243 (1973) 218.
36 Smets, L A & Ley, L de, J cell physiol 84 (1974) 343.
37 Collard, J G & Smets, L A, Exptl cell res 86 (1974) 75.
38. Cohen, H J, Anal biochem 53 (1973) 208.
39. Gordon, J A & Marquardt, M D, Biochim biophys acta 332 (1974) 136.
40 Burger, M M, Fed proc 32 (1973) 91.
41 Raff, M C & Petris, S de, Fed proc 32 (1973) 48
42. Rowlatt, C & Wicker, R, J ultrastruct res 40 (1972) 145
43. Huet, Ch & Herzberg, M, J ultrastruct res 42 (1973) 186
44. Temmink, J H M & Spiele, H, J cell biol 61 (1974) 548
45 Bretton, R, Ternynck, T & Avrameas, S, Exptl cell res 71 (1972) 145.
46. Huet, Ch & Garrido, J, Exptl cell res 75 (1972) 523.

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Paper VI

SURFACE MORPHOLOGY AND AGGLUTINABILITY WITH CONCANAVALIN A IN NORMAL AND TRANSFORMED MURINE FIBROBLASTS

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ABSTRACT

The surface morphology of attached and suspended normal and transformed fibroblasts has been studied with the scanning electron microscope. Normal murine fibroblasts (3T3) grow in vitro with widely extended leading lamellae. During most parts of the cell cycle the surfaces of these cells are practically free of microvilli. When the cells round up for mitosis, their cell surfaces become adorned with many microvilli. In contrast, simian virus 40-transformed fibroblasts (SV3T3) grow more compact, and their cell surfaces remain smooth throughout the life cycle. When confluent 3T3 and SV3T3 cells are suspended with ethylenediaminetetraacetic acid (EDTA) for agglutination assays, similar differences in surface morphology are found: 3T3 cells always bear many microvilli, whereas most SV3T3 cells are essentially free of microvilli. The addition of concanavalin A (Con A) does not influence the surface morphology of the suspended cells. The morphological differences described here may be important for the agglutination process of the normal and transformed 3T3 cells, because they affect the real cell surface area and thus the density of Con A-binding sites.

The plant lectin concanavalin A (Con A) binds specifically to certain carbohydrates of the cell membrane and agglutinates transformed cells generally more easily than their normal counterparts (7, 15). For that reason many investigators have attempted lately to correlate the agglutinability by Con A with the number and distribution of Con A-binding sites on the cell membrane Although a correlation between agglutination and the amount of cell-bound Con A has sometimes been found (19, 20), other reports indicate that differences and changes in agglutination can occur without concomitant changes in the amount of cell-bound Con A per unit cell surface area (3, 8, 14, 21)

Most recently, differences in the mobility of the binding sites in the plane of the membrane have been suggested as the cause for differences in agglutination between normal and transformed cells The theory implies that cross-linking agents, such as antibodies or lectins, can induce clustering of their specific binding sites in the membrane to a degree determined by the fluidity of the membrane and correlated with transformation (4, 16, 17, 18, 25, 26). In spite of the large amount of evidence for the occurrence of clustering and its occasional correlation with transformation, other data suggest that differences in clustering and agglutinability do not always coincide (22, 31, 32)

Thus, additional factors seem to play a role in the agglutination process, and therefore it ap-

peared useful to consider other parameters that change with transformation and agglutinability One of these parameters is the gross surface morphology of normal and transformed cells (11) Furthermore it is known that, during the cell cycle, agglutinability (29) and surface morphology (23) change considerably Therefore, we studied changes in surface morphology both during the cell cycle and after suspending cells for agglutination assays in order to find a correlation with changes in agglutination of these cells as determined in previous studies (11, 29) The relevance of our data for the general agglutination theory is discussed

MATERIALS AND METHODS

Tissue Culture

The cells used in our experiments were obtained commercially from Flow Laboratories, Inc , Rockville, Md as research grade normal 3T3 fibroblasts (Swiss albino ATCC [American Type Culture Collection, Rockville, Md] cell repository no CCL 92) and the simian virus 40-transformed (SV3T3) cells In indirect immunofluorescence tests for the presence of T antigen, all SV3T3 cells were found to be positive The saturation density of 3T3 cells in medium containing 10% newborn calf serum (NBS) is approximately 70,000 cells per cm^2, and all cells are then resting G_0 cells (9) The midpoint agglutination (mpa) with Con A of these 3T3 cells is reached at approximately 1,000 μg/ml Con A (11, 29) Transformed cells reach a maximum density of approximately 500,000 cells per cm^2 At higher densities the cells tend to detach from the growth substrate The transformed 3T3 cells reach mpa at approximately 15 μg/ml Con A (11, 29)

The two cell lines were also used in our laboratory for studies on cell cycle dependent (11, 29) and density dependent (30) Con A-mediated agglutinability and on redistribution of Con A-binding sites on the plasma membrane (32)

Cells were grown in plastic Petri dishes (10 cm) containing 10 ml of Dulbecco's modified Eagle's medium, supplemented with 10% NBS and antibiotics, in a humidified CO_2 incubator at 37°C The cells were seeded at a density of 50,000 per ml of nutrient medium

Synchronization Procedure

Cells at the G_1/S boundary were acquired by synchronization in excess thymidine (7 mM) after stationary SV3T3 or confluent, contact-inhibited 3T3 cells were plated Cells in S, G_2, and M were obtained from cultures synchronized with excess thymidine by washing them twice with prewarmed regular medium, and leaving them for 4 h, 8 h, and 10 h, respectively G_2 cells were also obtained by irradiation with 1800 R of X rays After 24 h, more than 90% of these cells had accumulated at the radiation-induced block in G_2 These synchronization procedures did not specifically alter the cell morphology, as could be ascertained by comparison with asynchronous cultures

Normal cells were sometimes synchronized by serum stimulation cells grown to confluency in medium with 3% serum were stimulated with medium containing 25% serum In this manner, approximately 75% of the cells were induced to go through the complete cell cycle Samples were taken at different times after serum stimulation

Samples of all preparations of synchronized cells were prepared for impulse cytophotometry, and the degree of synchronization was checked Details of the preparation of cells for impulse cytophotometry have been published elsewhere (9) Samples were used for scanning electron microscopy when at least 70 80% of the cells were in the required phase of the cell cycle

Scanning Electron Microscopy

For scanning electron microscopy, 3T3 and SV3T3 cells were grown on cover slips, rinsed in PBS, and fixed for 30 min in 2 5% buffered glutaraldehyde The cover slips with attached cells were put in a holder that had been specially designed to fit the pressure vessel of a critical point drying apparatus For dehydration, this holder was left in each of a graded series of ethanol solutions (50% 70% 90% 100% 100%) for 5 min and twice in 100% amyl acetate for 10 min After the holder was quickly transferred to the pressure vessel, the cells were dried according to the method of Anderson (2), using liquid CO_2 The dried cells on glass were mounted on stubs and covered with gold in a Balzers freeze-etching apparatus

Suspended cells were obtained by detaching cells growing in Petri dishes with 1×10^{-3} M ethylenediaminetetraacetic acid, EDTA, in Ca^{++}- and Mg^{++}-free PBS and washing them twice in PBS containing Ca^{++} and Mg^{++} The cells were layered on cover slips with attached confluent 3T3 cells by adding 2 ml of the suspension (1×10^6 cells/ml) After 10-min incubation at 37°C in PBS, most of the suspended cells had become attached to the underlying monolayer, and the cover slips were fixed and dried as described above

Treatment of cells with Con A at room temperature occurred in 1 ml of cell suspension (2×10^6 cells/ml) by adding 1 ml of Con A to reach the desired final concentration (up to 1000 μg/ml) After 20-min incubation, the cells were layered on cover slips All material was studied in a Cambridge Stereoscan microscope

RESULTS

Cells in Situ

Recently, Porter et al (23) have shown changes in surface morphology of CHO cells during the cell cycle, and a cell cycle dependent agglutinability

with Con A has been found in transformed fibroblasts (29). Therefore, we studied changes in surface morphology of 3T3 and SV3T3 cells during the cell cycle in an attempt to find differences that might influence the agglutination process of these cells.

To that purpose, asynchronous and synchronized normal and transformed 3T3 cells were prepared for scanning electron microscopy. The degree of synchronization was checked with an impulse cytophotometer Fig 1 shows a representative example of impulse cytophotometric graphs of SV3T3 cells, indicating the degree of synchronization routinely obtained with both cell types.

Normal subconfluent 3T3 cells in late G_1, S, and G_2 had widely spread, flat leading lamellae (1). At these stages, all cells had practically no microvilli or zeiotic blebs (Fig. 2 *a* and *b*), and the cells were morphologically indistinguishable from one another. Only impulse cytophotometry showed that the cells were synchronized as indicated in the legend. In late G_2 (just before mitosis) the cells began to retract the leading lamellae, and their surfaces showed many microvilli of varying length (Fig. 2 *c*) and sometimes zeiotic blebs (not shown). Slender extensions at the sides of the cells were also found and considered to be developing retraction fibrils (12) (Fig. 2 *c*, arrow). With the decreasing size of the cell surfaces, the number of microvilli seemed to increase, and the retraction fibrils on the sides of the cells became longer After completely rounding up for mitosis, the cells remained attached to the growth substrate by a number of retraction fibrils only (Fig 2 *d*). After cytokinesis, the two daughter cells immediately began to spread over the substrate along the retraction fibrils This process continued in early G_1, and during that phase of the cell cycle the number of microvilli gradually decreased (Fig 2 *e*). If zeiotic blebs had been present, they also tended to disappear during or after mitosis, and by late G_1 the cells had become fully expanded with smooth surfaces. Resting confluent 3T3 cells (G_0) did not differ markedly from subconfluent 3T3 cells in late G_1, S, or G_2 with respect to the size of the leading lamellae Their surfaces were not always completely free of microvilli (Figs 2 *f* and 4 *a*, Fig 6, underlying cells), but, if present, the microvilli were much shorter than on subconfluent 3T3 cells in mitosis

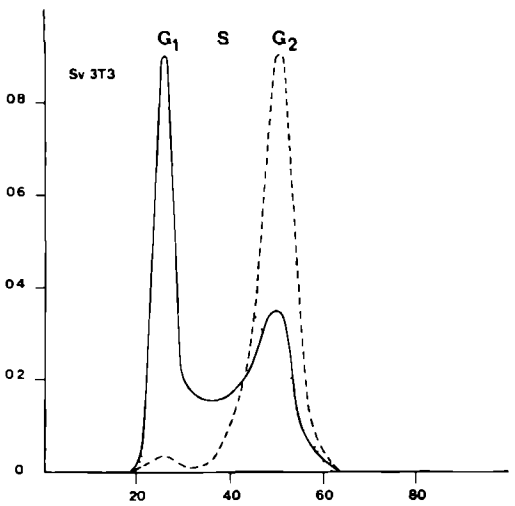

FIGURE 1 Impulse cytophotometric graph of distribution of SV3T3 cells in the cell cycle Abscissa channel number (increasing amount of DNA/cell) Ordinate cells per channel ($\times 10^4$) (——), asynchronous culture, (· ·), thymidine-synchronized culture in S, (-----), culture in G_2 after X-ray irradiation.

SV3T3 cells synchronized in late G_1, S, and G_2 resembled their normal parent cells somewhat, especially in subconfluent and confluent cultures, but they were smaller and less widely spread over the underlying glass (Fig 3 *a* and *b*). The differences in morphology and in growth pattern between normal and transformed 3T3 cells became more clearly visible after the cells had reached confluent and superconfluent densities (compare Figs 4 *a* and 5 *a*). The surfaces of SV3T3 cells in late G_1, S, and G_2 were completely smooth. In late G_2, the cells began to contract, but, in contrast to 3T3 cells, the SV3T3 cells did not develop many microvilli on the upper surfaces (Fig. 3 *c*, arrow *1*). Sometimes, zeiotic blebs could be found, but in most cases the upper cell surfaces were free of extensions (Fig 3 *c*, *insert*). Only shallow ridges and small folds were often present, perhaps as traces of withdrawn leading lamellae With the decreasing size of the cells, retraction fibrils at the periphery of the cells increased in number and length. During mitosis, the cell surfaces remained practically free of microvilli (Fig 3 *c*, arrow *2*) After cytokinesis, the cells began to spread, and during early G_1 the number of retraction fibrils decreased with the progressive flattening of the cells over the underlying surface

Thus, the scanning electron micrographs of 3T3 and SV3T3 cells *in situ* revealed two major differences between normal and transformed cells (*a*) transformed cells are less widely extended over the growth substrate than normal cells and (*b*)

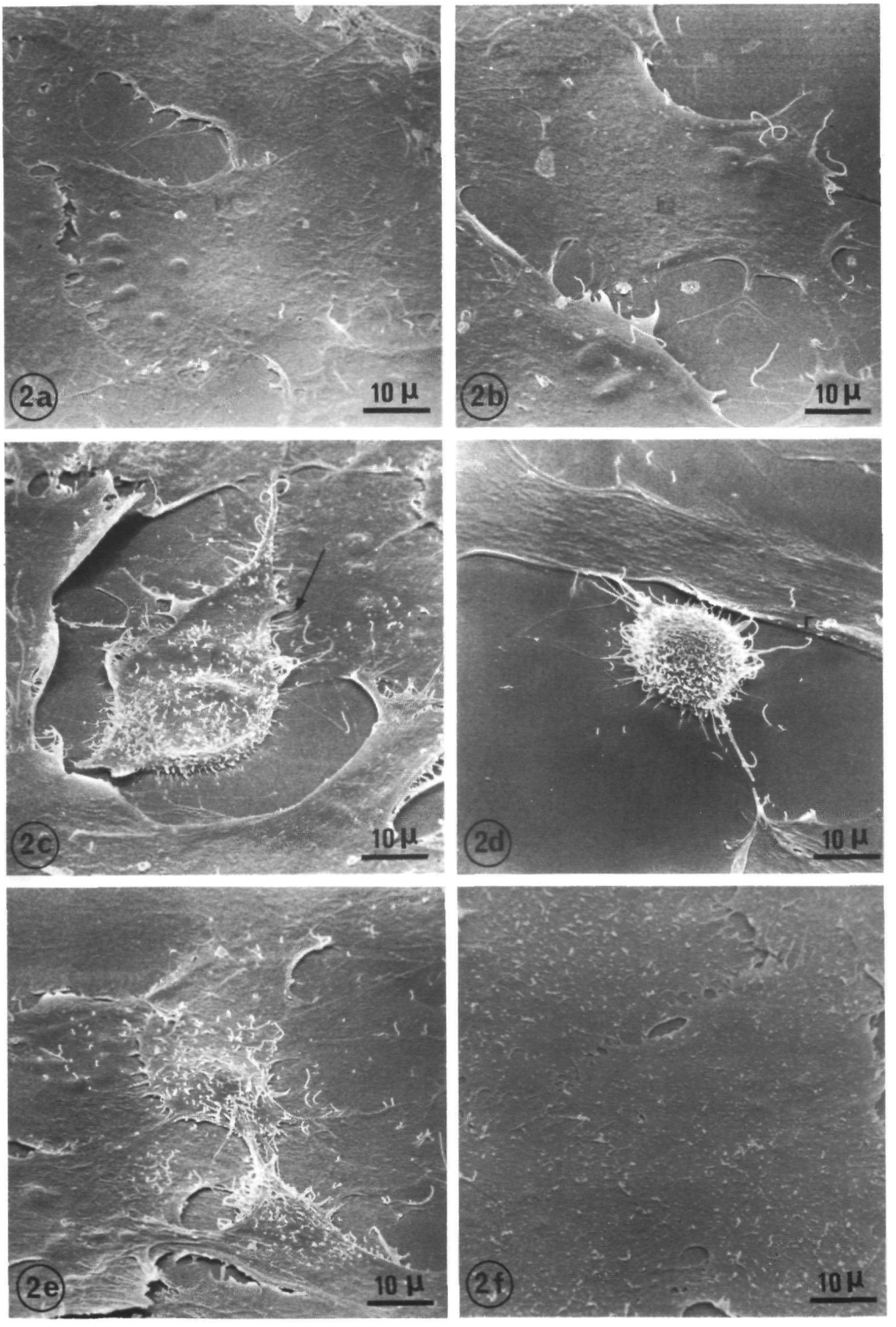

FIGURE 2 Subconfluent, normal 3T3 cells *in situ*. (*a*) Late G_1 cells at thymidine block; (*b*) S cells grown for 4 h after release of thymidine block; (*c*) Late G_2 cell in field of G_2 cells, grown for 8 h after release of thymidine block; (*d*) Mitotic cell in culture grown for 10 h after release of thymidine block; (*e*) Early G_1 cells in same culture as (*d*); and (*f*) Confluent culture (G_0 cells).

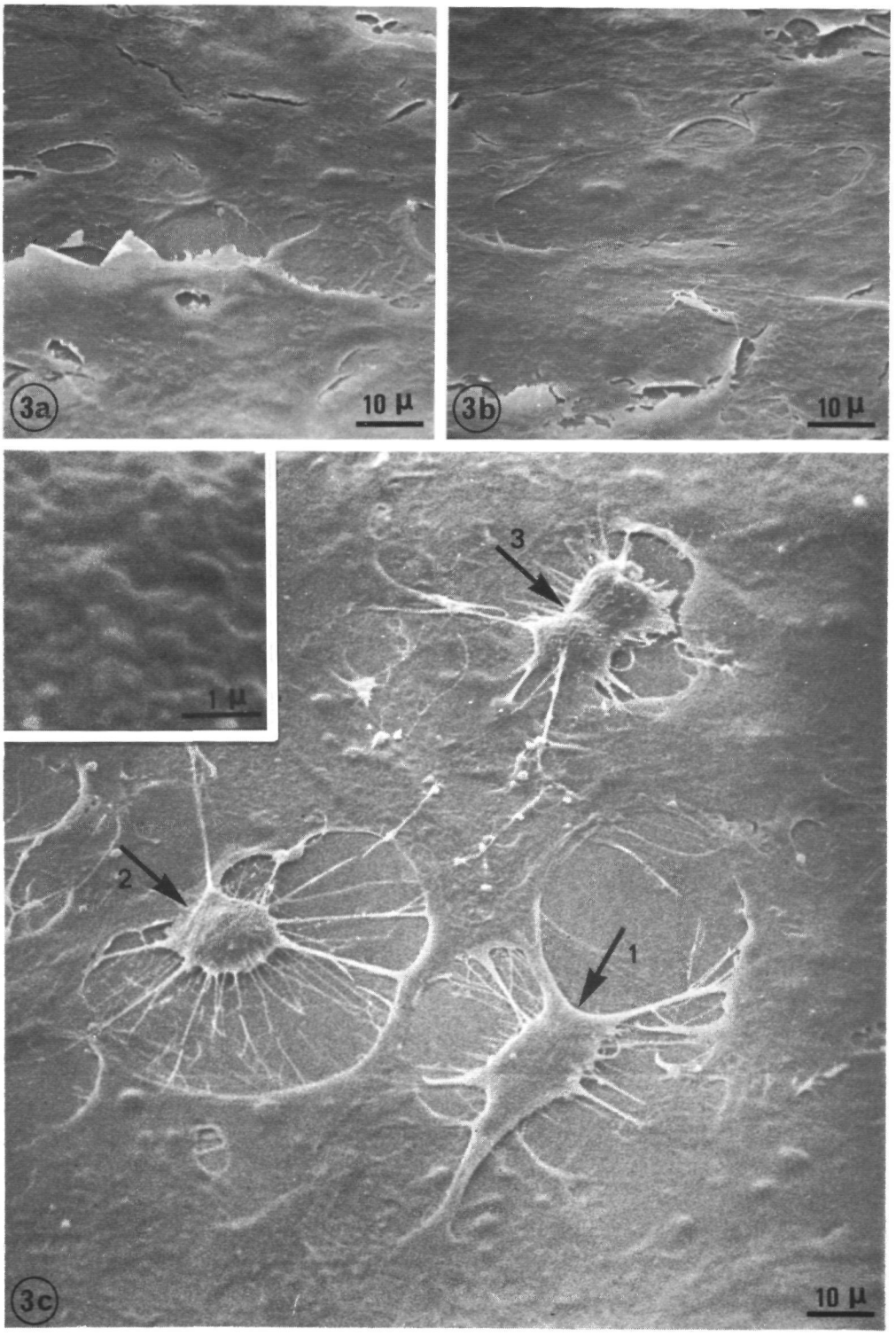

FIGURE 3 Almost confluent, transformed 3T3 cells *in situ*. (*a*) Late G_1 cells at thymidine block; (*b*) S cells grown for 4 h after release of thymidine block; and (*c*) Late G_2 cell (arrow *1*), mitotic cell (arrow *2*), and early G_1 cell (arrow *3*) in culture grown for 10 h after release of thymidine block; the *insert* shows part of the cell surface of a mitotic cell at higher magnification.

whereas 3T3 cells have a great number of microvilli on the upper cell surfaces during late G_2, M, and early G_1, SV3T3 cells remain free of microvilli throughout their entire life cycle

Cells Detached with EDTA

To determine whether the morphological differences between 3T3 and SV3T3 cells *in situ* are relevant for the agglutination process, we investigated the same cells in suspended form since suspended cells are used in the agglutination assays.

Fig. 4 *a–d* show the changes in surface morphology of confluent 3T3 cells during detachment from the growth substrate with EDTA. As mentioned above, these cells were widely spread and the surfaces occasionally bore few short microvilli (Fig. 4 *a*). The edges of adjacent cells were in complete contact with one another, except where slight shrinkage during the drying procedure had broken the contact locally. Impulse cytophotometry of these cells indicated that no S and G_2 cells were present (9). After incubation with EDTA for 5 min at 25°C, the cells began to withdraw their leading lamellae (Fig. 4 *b*). At this stage, no changes on the cell surface were yet observed compared to untreated control cells. After incubation with EDTA under the same conditions for 7 min, the leading lamellae were almost fully withdrawn. Many microvilli of different lengths began to appear on the cell surface (Fig. 4 *c*). A few short retraction fibrils became also visible at this stage. After treatment with EDTA for 10 min, the cells were practically rounded up (Fig. 4 *d*). Many microvilli were seen on the surfaces of these cells. Contact with the underlying substrate was maintained by slender retraction fibrils resembling morphologically some of the longer microvilli Sometimes, blebs might also appear on the surfaces of some cells during rounding up. Fig. 4 *e* and *f* show the cells brought into suspension as described and layered on cover slips with attached confluent 3T3 cells Most suspended 3T3 cells had surfaces adorned with many microvilli. The microvilli might vary in length and, to a certain extent, in number, but they were always numerous.

Fig. 5 *a–d* illustrate the detaching of superconfluent SV3T3 cells with EDTA Untreated SV3T3 cells had no extensions on the cell surfaces (Fig 5 *a*). After treatment with EDTA for 3 min at 25°C, the small leading lamellae began to withdraw, but the surface structure remained unaltered (Fig. 5 *b*). The leading lamellae were almost completely withdrawn after incubation with EDTA for 5 min, and a number of retraction fibrils became visible (Fig 5 *c*) Practically no microvilli were visible on the surfaces of most cells, but the cell surfaces showed some shallow folds and ridges. Incubation with EDTA for 10 min resulted in rounded cells attached to the substrate with some retraction fibrils (Fig 5 *d*). Most of the cells had relatively smooth surfaces free of microvilli but with folds and creases. On some other cells, varying numbers of rather short microvilli were present. When SV3T3 cells were suspended and layered on cover slips with confluent 3T3 cells, the transformed cells showed a range of appearances (Fig 5 *e* and *f*). Most cells were smooth without microvilli, but cells with varying numbers of microvilli were also found. The smooth surface in most cases had some folds and ridges. These were less clearly seen on cells with microvilli.

Because of the variation in surface morphology of individual cells and the resulting difficulty in demonstrating this in a few micrographs, we introduced a double-blind test to determine the composition of each sample suspended 3T3 and SV3T3 cells were investigated under code numbers, and approximately 25 pictures were randomly taken of each sample of four different experiments. The coded photographs of several samples were put together, and a number of persons were asked to sort the cells into three categories according to the number and length of the microvilli on the surfaces (rough, intermediate, and smooth) The results are shown in Fig. 7. A marked difference was found consistently in the average surface morphology between 3T3 and SV3T3 cells, that is, between cells with low and high agglutinabilities.

Suspended Cells Treated with Con A

Experiments on lymphoblasts had shown that their surface morphology may be greatly influenced by the addition of Con A in concentrations necessary for agglutination. Upon addition of Con A, the microvilli present on these cells tend to flatten over the cell surfaces and seem to be resorbed by the cells (Collard, J. G., and J. H. M. Temmink. Submitted for publication.). Because of our interest in the relation between surface morphology and agglutinability, we investigated the effect of Con A on the surface morphology of suspended 3T3 and SV3T3 cells after incubation with different concentrations of Con A.

However, the surface morphology of both 3T3

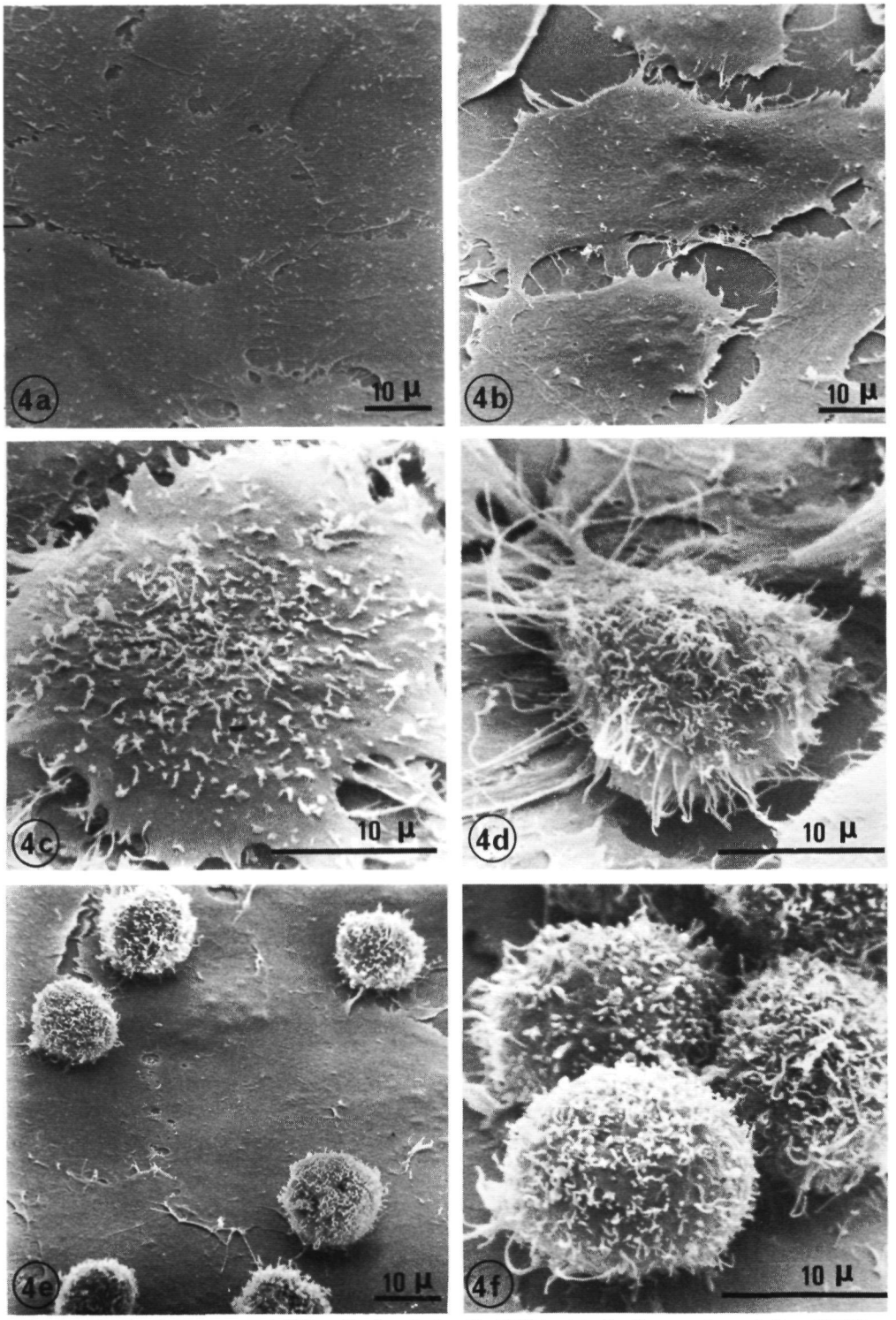

FIGURE 4 Confluent, normal 3T3 cells detached with EDTA. (*a*) Confluent culture before EDTA addition; (*b*) Culture 5 min after EDTA addition; (*c*) Culture 7 min after EDTA addition; (*d*) Culture 10 min after EDTA addition; and (*e* and *f*) EDTA-detached cells layered on confluent 3T3 cells.

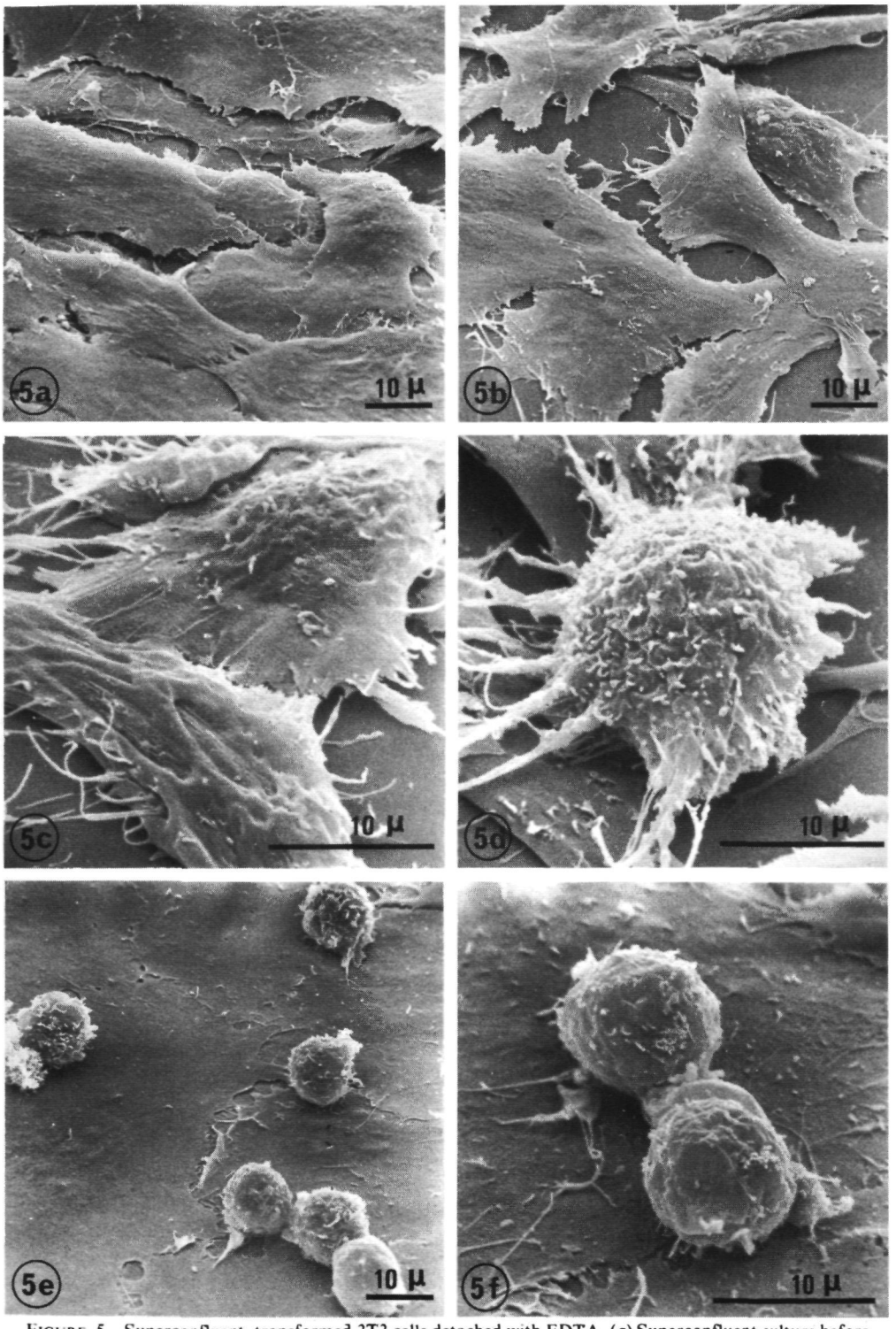

FIGURE 5 Superconfluent, transformed 3T3 cells detached with EDTA. (*a*) Superconfluent culture before EDTA addition; (*b*) Culture 3 min after EDTA addition; (*c*) Culture 5 min after EDTA addition; (*d*) Culture 10 min after EDTA addition; and (*e* and *f*). EDTA-detached cells layered on confluent 3T3 cells.

and SV3T3 cells incubated with different concentrations of Con A (up to 1,000 μg/ml) was not affected (Figs. 6 and 7). In number and length, the microvilli were similar to those on suspended cells not exposed to Con A.

DISCUSSION

The present investigation on the surface morphology of normal and transformed 3T3 cells revealed two conspicuous differences. (*a*) Normal cells have widely spread leading lamellae in subconfluent and confluent cultures, whereas transformed cells in subconfluent cultures have relatively small leading lamellae that decrease further in size when the cultures reach confluent and superconfluent densities. This difference in size has been described before in light microscope studies (5) and in studies on replicas of these cells (11). It partially agrees with a recent scanning electron microscope study on attached normal and transformed BALB/3T3 cells (24), although the SV40-transformed BALB/3T3 cells are much more spindle-shaped than our SV40-transformed Swiss 3T3 cells. In addition, the SV40-transformed BALB/3T3 cells, but not the spontaneously transformed BALB 3T3 cells, resemble our SV40-transformed Swiss 3T3 cells in the virtual absence of microvilli from the surfaces (24).

(*b*) Synchronized cultures of 3T3 and SV3T3 cells differ clearly in the morphological changes that occur around the mitotic process. Whereas most SV3T3 cells have practically no microvilli

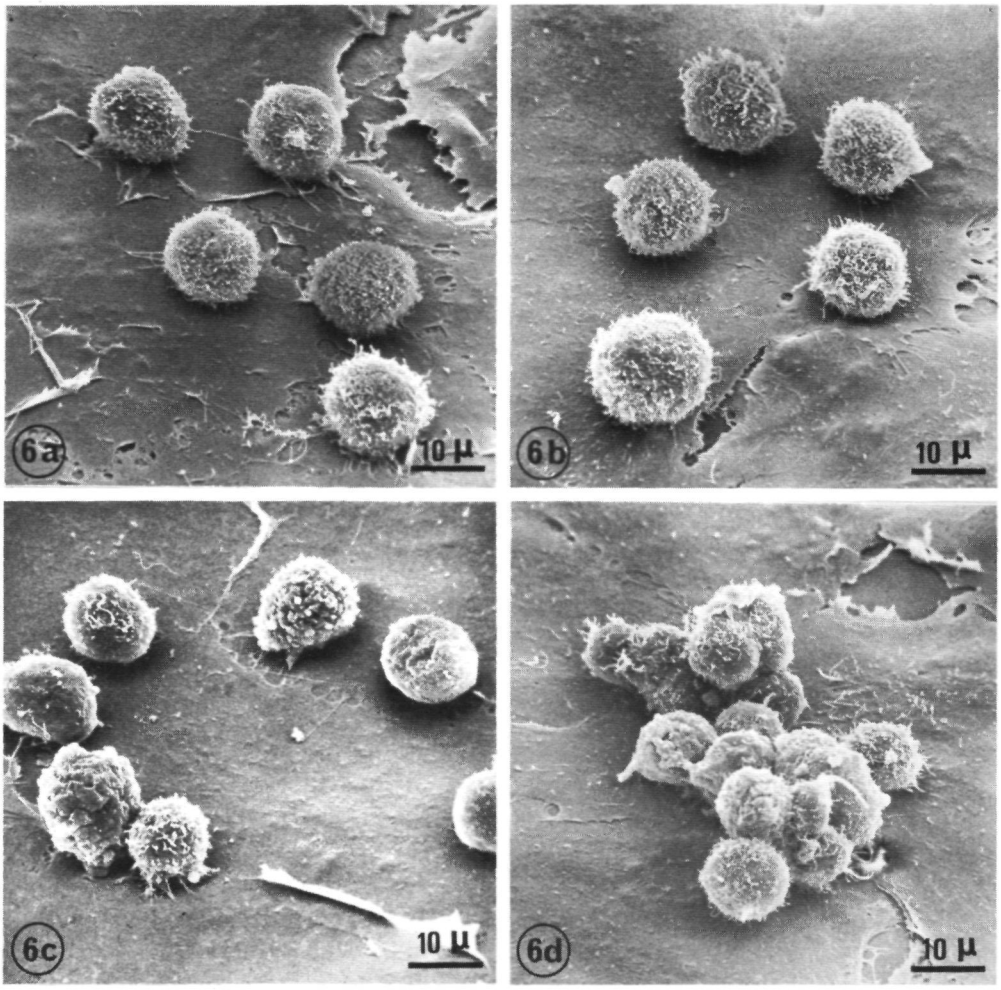

FIGURE 6 Suspended 3T3 and SV3T3 cells with and without addition of Con A (500 μg/ml). (*a*) 3T3 cells without Con A; (*b*) 3T3 cells with Con A; (*c*) SV3T3 cells without Con A; and (*d*) SV3T3 cells with Con A.

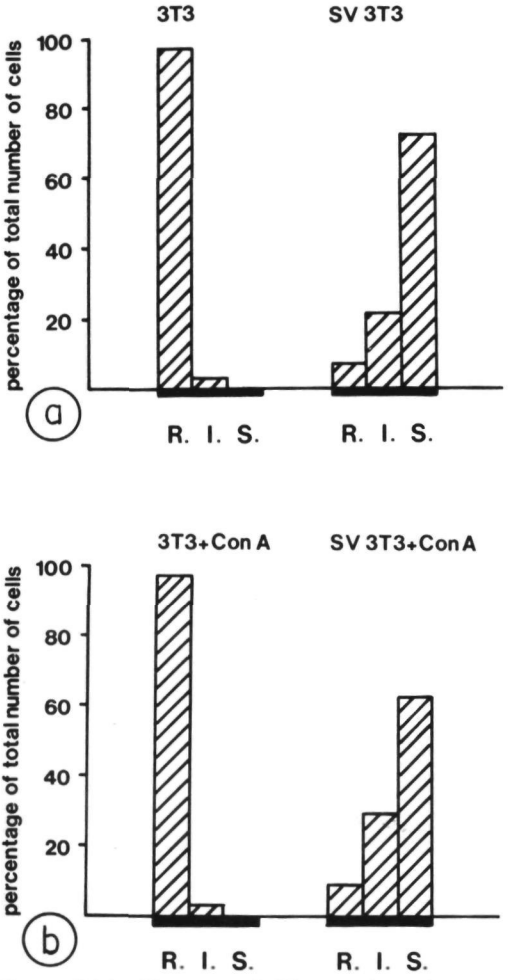

FIGURE 7 (*a*) Histogram of differences in surface morphology between 3T3 and SV3T3 cells. R, rough (with many microvilli); I, intermediate; and S, smooth (without microvilli). (*b*) Histogram of differences in surface morphology between 3T3 and SV3T3 cells at 20 min after Con A addition (500 μg/ml). R, rough (with many microvilli); I, intermediate; and S, smooth (without microvilli).

around mitosis, normal 3T3 cells always have many microvilli in late G_2, M, and early G_1. These changes in surface morphology during the cell cycle of 3T3 cells are in general agreement with the observations of Porter et al. on CHO cells (23). However, in contrast with the observations on CHO cells (27), the variation in the number of microvilli during the cell cycle of SV3T3 cells was greater in subconfluent than in confluent cultures. SV3T3 cells in confluent and superconfluent cultures had only retraction fibrils, and microvilli were absent from the cell surfaces during mitosis.

The difference in size between spread 3T3 and SV3T3 cells *in situ* seems to be reflected in detached cells in differences in the number of microvilli similar to the differences in number of microvilli in spontaneously contracting cells *in situ* during mitosis. This might perhaps be considered to indicate that microvilli develop on cells as a means of "storing" surplus membrane material, as suggested before (13).

The presence or absence of zeiotic blebs has been mentioned in the present paper without much emphasis. We do not know what conditions during growth or handling of the cells determine whether zeiotic blebs arise. Since in our material some mitotic or suspended cells had zeiotic blebs only occasionally, we considered the blebs to be artifacts. Because the formation of blebs is not known to interfere with the formation of microvilli and because blebbing does not influence the viability of the cells, we have neglected the phenomenon as irrelevant for the subject of this paper.

The variation in morphological appearance, especially of individual suspended transformed cells, as demonstrated in the histograms (Fig. 7), is probably due to a variable effect of EDTA on the cells, and is also, perhaps, a result of the different phases of the cell cycle in which the cells are suspended.

Treatment of suspended cells with Con A as in agglutination assays did not influence markedly the morphological appearance of suspended normal and transformed 3T3 cells. Our data indicate that the described morphology of our suspended fibroblasts reflects the surface morphology of these cells in agglutination assays.

The data presented here show a large difference in surface morphology between normal and transformed cells, probably as a result of the large difference in size between these cells *in situ* and the difference in capacity to absorb excess membrane. This difference in morphology affects the cell surface area determinations made in order to correlate the density of Con A-binding sites with agglutinability. As yet, cell surface areas of suspended normal and transformed 3T3 cells have been determined on the assumption that both cell types are perfect spheres in suspension (5, 15, 19). However, our data indicate that the cell surface areas of normal suspended cells are much larger than those of transformed cells, due to the microvilli. Because equal numbers of Con A molecules are bound to normal and transformed cells (3, 5, 8, 21), and because our preliminary data on Con A-binding site distribution on suspended cells

indicate that microvilli have essentially the same number of Con A-binding sites as cell membrane areas between microvilli, the density of Con A-binding sites must be much greater on transformed than on normal cells The quantitative aspects of the influence of surface morphology on the number of Con A-binding sites have been treated in a separate paper (10) The difference in the density of Con A-binding sites may also play a role in the difference in degree of clustering of these sites on normal and transformed cells, as originally observed by Nicolson (16) and Singer and Nicolson (28), and may be the main cause of the different agglutination responses of normal and transformed 3T3 cells, as originally suggested by Burger (6)

We are presently investigating whether the described morphological changes in attached cells during the cell cycle are reflected in the surface morphology of synchronized suspended cells in correlation with described changes in the agglutinability (29) Furthermore, it is of interest how brief proteolytic treatment affects the surface morphology of suspended normal cells in relation to the described increase in agglutinability (6)

ADDENDUM

After submission of this manuscript, a paper by Willingham and Pastan[1] appeared with data that seemed to completely contradict the results presented in our report The cells of their transformed murine fibroblast line (L 929), *in situ* as well as in suspension, were adorned with many microvilli, whereas their normal cells (3T3 4) were free of these surface extensions when *in situ* but developed them when rounding off spontaneously (mitosis) or by trypsin treatment The L 929 could be made free of microvilli by dibutyryl cyclic AMP, a drug that also converts the growth pattern and agglutinability of these cells to those of nontransformed cells

However, additional research may prove the above discrepancies to be due to differences in the cell material used This seems likely when the results of Porter et al (24) are also taken into account The absence of microvilli from their SV40-transformed BALB/3T3 resembles that on our transformed Swiss 3T3, whereas the results on their spontaneous transformants (e g S2 4) would seem to substantiate Willingham and Pastan's data

In addition, we agree with Nicolson[2] that the presence or absence of microvilli alone does not explain the differences in agglutinability our smooth SV3T3 cells agglutinate very well with Con A, but so do our lymphoblasts (Raji and EB_3) that have many microvilli, whereas normal peripheral lymphocytes with many microvilli do not agglutinate with Con A (Collard, J G , and J H M Temmink Submitted for publication)

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REFERENCES

1 Abercrombie, M , Joan E M Heaysman, and Susan M Pegrum 1970 The locomotion of fibroblasts in culture I Movement of the leading edge *Exp Cell Res* **59**:393 398

2 Anderson, T F 1951 Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope *Trans N Y Acad Sci* **13**.130–134

3 Arndt-Jovin, D J and P Berg 1971 Quantitative binding of ^{125}I-Concanavalin A to normal and transformed cells *J Virol* **8**:716–721

4 Barnett, R E , L T Furcht, and R E Scott 1974 Differences in membrane fluidity and structure in contact-inhibited and transformed cells *Proc Natl Acad Sci U S A* **71**: 1992 1994

5 Ben-Bassat, H , M Inbar, and L Sachs 1971 Changes in the structural organization of the surface membrane in malignant cell transformation *J Membr Biol* **6**:183 194

6 Burger, M M 1969 A difference in the architecture of the surface membrane of normal and virally transformed cells *Proc Natl Acad Sci U S A* **62**:994 1001

7 Burger, M M 1973 Surface changes in transformed cells detected by lectins *Fed Proc* **32**:91 101

8 Cline, M J and D C Livingston 1971 Binding of ^{3}H Concanavalin A by normal and transformed cells *Nat New Biol* **232**:155–156

[1] Willingham, M C , and Ira Pasten 1975 Cyclic AMP modulates microvillus formation and agglutinability in transformed and normal mouse fibroblasts *Proc Natl Acad Sci U S A* **72**:1263

[2] Nicolson, G L 1974 The interactions of lectins with animal cell surfaces *Int Rev Cytol* **39**:89 190

9 COLLARD, J G, and L A Smets 1974 Effect of proteolytic inhibitors on growth and surface architecture of normal and transformed cells *Exp Cell Res* **86**:75–80

10 COLLARD, J G, and J H M TEMMINK 1975 Differences in density of Concanavalin A binding sites due to differences in surface morphology of suspended normal and transformed 3T3 cells *J Cell Sci* **19**:21–32

11 COLLARD, J G, J H M TEMMINK, and L A SMETS 1975 Cell cycle dependent agglutinability, distribution of Concanvalin A binding sites and surface morphology of normal and transformed fibroblasts *In* Concanavalin A Proceedings of an International Symposium on Concanavalin A (T K Chowdhury and A K Weiss, Editors) Plenum Publishing Corporation, New York 221–244

12 DALEN, H, and P W TODD 1971 Surface morphology of trypsinized human cells in vitro *Exp Cell Res* **66**:353–361

13 FOLLETT, E A C, and R D GOLDMAN 1970 The occurrence of microvilli during spreading and growth of BHK21/C13 fibroblasts *Exp Cell Res* **59**:124–136

14 INBAR, M, H BEN-BASSAT, and L SACHS 1972 Membrane changes associated with malignancy *Nat New Biol* **236**:3–4 and 16

15 INBAR, M, and L SACHS 1969 Interaction of the carbohydrate-binding protein Concanavalin A with normal and transformed cells *Proc Natl Acad Sci U S A* **63**:1418 1425

16 NICOLSON, G L 1971 Difference in topology of normal and tumour cell membranes shown by different surface distributions of ferritin-conjugated Concanavalin A *Nat New Biol* **233**:244–246

17 NICOLSON, G L 1972 Topography of membrane Concanavalin A sites modified by proteolysis *Nat New Biol* **239**:193 197

18 NICOLSON, G L 1973 Temperature-dependent mobility of Concanavalin A sites on tumour cell surfaces *Nat New Biol* **243**:218–220

19 NOONAN, K D, and M M BURGER 1973 Binding of ^{3}H-Concanavalin A to normal and transformed cells *J Biol Chem* **248**:4286–4292

20 NOONAN, K D, A J LEVINE, and M M BURGER 1973 Cell cycle-dependent changes in the surface membrane as detected with ^{3}H-Concanavalin A *J Cell Biol* **58**:491–497

21 OZANNE, B, and J SAMBROOK 1971 Binding of radioactively labeled Concanavalin A and wheat germ agglutinin to normal and virus-transformed cells *Nat New Biol* **232**:156–160

22 PETRIS, S DE, M C RAFF, and L MALLUCCI 1973 Ligand-induced redistribution of Concanavalin A receptors on normal, trypsinized and transformed fibroblasts *Nat New Biol* **244**:275–278

23 PORTER, K, D PRESCOTT, and J FRYE 1973 Changes in surface morphology of chinese hamster ovary cells during the cell cycle *J Cell Biol* **57**:815 836

24 PORTER, K D, G J TODARO, and V FONTE 1973 A scanning electron microscope study of surface features of viral and spontaneous transformants of mouse Balb/3T3 cells *J Cell Biol* **59**:633 642

25 POSTE, G, and P REEVE 1974 Increased mobility and redistribution of Concanavalin A receptors on cells infected with Newcastle disease virus *Nature (Lond)* **247**:469 471

26 ROSENBLITH, J Z, T E UKENA, H H YIN, R D BERLIN, and M J KARNOVSKY 1973 A comparative evaluation of the distribution of Concanavalin A binding sites on the surfaces of normal, virally-transformed, and protease-treated fibroblasts *Proc Natl Acad Sci U S A* **70**:1625 1629

27 RUBEN, R W, and L P EVERHART 1973 The effect of cell-to-cell contact on the surface morphology of chinese hamster ovary cells *J Cell Biol* **57**:837 844

28 SINGER, S J, and G L NICOLSON 1972 The fluid mosaic model of the structure of cell membranes *Science (Wash D C)* **175**:720 731

29 SMETS, L A, and L DE LEY 1974 Cell cycle dependent modulation of the surface membrane of normal and SV-40 transformed 3T3 cells *J Cell Physiol* **84**:343 348

30 SMETS, L A, L DE LEY, and J G COLLARD 1975 Contact mediated changes in cytoagglutination *Exp Cell Res* In press

31 SMITH, S B, and J-P REVEL 1972 Mapping of Concanavalin A binding sites on the surface of several cell types *Dev Biol* **27**:434–441

32 TEMMINK, J H M, J G COLLARD, H SPITS, and E ROOS 1975 A comparative study of four cytochemical detection methods of Concanavalin A binding sites on the cell membrane *Exp Cell Res* **92**:307 321

Paper VII

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Printed in Great Britain

DIFFERENCES IN DENSITY OF CONCANAVALIN A-BINDING SITES DUE TO DIFFERENCES IN SURFACE MORPHOLOGY OF SUSPENDED NORMAL AND TRANSFORMED 3T3 FIBROBLASTS

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SUMMARY

Calculations of the density of Concanavalin A (Con A)-binding sites on normal and transformed fibroblasts have, as yet, been based on the unproven assumption that suspended cells are smooth spheres. We studied the surface morphology of suspended normal and transformed fibroblasts with scanning and transmission electron microscopes, and found a large difference in surface morphology between suspended normal and transformed 3T3 cells. When this difference in surface morphology was taken into account, the estimated cell surface area of normal 3T3 cells was approximately seven times larger than that of transformed 3T3 cells. Since equal numbers of ^{3}H-Con A molecules are bound on normal and transformed cells, the density of Con A-binding sites is approximately seven times greater on transformed than on normal 3T3 cells.

The difference in density of Con A-binding sites between normal and transformed fibroblasts might be sufficient to explain the difference in agglutination response, as originally suggested by Burger, and may also be the cause of the different degrees of clustering of Con A-binding sites on the plasma membrane of these cells.

INTRODUCTION

In order to explain the higher agglutinability of transformed as compared to normal fibroblasts, many reports concerning the number of Concanavalin A (Con A)-binding sites on the cell surface have been published (Nicolson, 1974). In most of these investigations equal numbers of Con A-binding sites on both cell types have been found (Arndt-Jovin & Berg, 1971; Cline & Livingston, 1971; Inbar, Ben-Bassat & Sachs, 1971; Ozanne & Sambrook, 1971). However, when the number of binding sites was calculated per unit of protein or cell surface area, 1–3 times more binding sites were generally found on transformed cells (Inbar & Sachs, 1969; Cline & Livingston, 1971; Ozanne & Sambrook, 1971; Ben-Bassat, Inbar & Sachs, 1971; Noonan & Burger, 1973).

Estimates of Con A-binding site density based on protein content or wet volume do not take into account that cell volume and cell surface area may fluctuate due to variations in water content. Estimates of cell surface area based on light-microscopic measurements of cell diameters or on wet volume determinations are based on the unproven assumption that suspended cells are smooth spheres.

In a previous report we suggested that differences in surface morphology between normal and transformed cells may play a role in their different agglutination responses (Collard, Temmink & Smets, 1975) Therefore we studied the effect of differences in surface morphology of suspended normal and transformed 3T3 cells on the actual surface area by scanning and transmission electron microscopy The results show great differences in the total cell surface area of suspended normal and transformed cells, indicating that transformed cells bind about seven times more molecules of Con A per unit of surface area than normal 3T3 cells These data are discussed in relation to current concepts of membrane changes after malignant transformation

MATERIALS AND METHODS

Tissue culture

Research grade normal (3T3) and simian virus 40-transformed (SV3T3) murine fibroblasts were obtained commercially from Flow Laboratories Cells were grown in plastic Petri dishes (ϕ 10 cm), containing 10 ml of Dulbecco's modified Eagle medium, supplemented with 10% newborn calf serum and antibiotics, in a humidified CO_2 incubator at 37 C The cells were seeded at a density of 5×10^4 cells per ml of nutrient medium The 3T3 cells (saturation density 7×10^4 cells per cm^2) were half maximally agglutinated at a Con A concentration of approximately 1000 μg ml and SV3T3 cells (saturation density 5×10^5 cells per cm^2) at approx 10–15 μg/ml (Smets & de Ley, 1974)

For the scanning electron-microscope studies, cells were grown on small coverslips in plastic Petri dishes (ϕ 5 cm) filled with 5 ml of nutrient medium

Electron microscopy

For scanning electron microscopy of attached cells, the cells were grown on coverslips, rinsed in phosphate-buffered saline (PBS, pH 7 2) and fixed for 30 min in 2 5 % buffered glutaraldehyde After dehydration in ethanol and amyl acetate, the cells were dried by the critical point method (Anderson, 1951), using CO_2 The dried cells on glass were mounted on stubs and covered with gold in a Balzer freeze-etching apparatus When detached cells were studied, they were suspended with 1×10^{-5} M EDTA in Ca^{2+} - and Mg^{2+} -free PBS, washed twice in PBS containing Ca^{2+} and Mg^{2+}, and layered on coverslips with a confluent monolayer of 3T3 cells After incubation for 10 min at 37 C in PBS, most of the suspended cells had become attached to the underlying monolayer and the coverslips were fixed and dried as described above All specimens were studied in a Cambridge Stereoscan Microscope

For transmission electron microscopy, suspended normal and transformed 3T3 cells were layered on carbon-coated coverslips with confluent 3T3 cells The cells were fixed in 2 5 % glutaraldehyde, dehydrated and embedded in Epon/Araldite They were separated from the glass, cross-sectioned, stained with uranyl acetate and lead hydroxide, and examined in a Philips 300 electron microscope Suspended cells were also investigated as a pellet Addition of Con A (up to 1000 μg) to the suspended cells did not influence their morphology

^{3}H-Con A preparation and binding

The ^{3}H-Con A was prepared by New England Nuclear by exposing Con A (Calbiochem) to 3 Ci of tritium gas for 2 weeks This material was purified in our laboratory by centrifugation and chromatography on Biogel-P-100 The purified ^{3}H-Con A reacted in the same way as unlabelled Con A in agglutination experiments and was indistinguishable in chromatographic behaviour Its specific activity was 2000 dpm/μg Con A

To measure binding of ^{3}H-Con A, cells were suspended with 1×10^{-3} M EDTA as described Samples of 1 ml with 1×10^6 cells were incubated for 20 min at room temperature with various concentrations of ^{3}H-Con A Next, the aliquots were sampled on filter disks, washed and counted in a liquid scintillation counter. Control experiments for the specificity of the binding

were performed by adding 0·05 M α-methyl-D-glucoside (α-MG) to the cells prior to addition of ^{3}H-Con A. In order to be able to compare our data with the data of other investigators, binding was calculated assuming a molecular weight of 55000 instead of 110000 (Nicolson, 1974). Thus we did not take into account the structure transitions of Con A at different temperatures (Huet, Lonchampt, Huet & Bernadac, 1974). In our binding experiments at room temperature we used what were probably predominantly tetramer Con A molecules. This may have affected the calculated absolute number of Con A-binding sites, but not the relative difference in Con A binding between normal and transformed cells.

Estimation of real cell surface area

Cell diameters were determined on at least 100 cells in the light microscope fitted with a micrometer eyepiece, or by measuring cell diameters on scanning electron micrographs. With these data the cell surface area was calculated on the assumption that the cells are smooth spheres (cell surface area I).

On the other hand, the enlargement of the cell surface by microvilli was estimated on transmission electron micrographs: the average length, thickness, and number of microvilli per cell were measured and the approximate surface area of the microvilli (considered as small cylinders) was calculated. This estimated surface area of the microvilli per cell was added to cell surface area I in order to obtain a value for the real cell surface, including the microvilli (cell surface area II).

RESULTS

Electron microscopy of attached and suspended cells

Scanning electron-microscope investigations on confluent attached normal and transformed fibroblasts showed smooth cells practically without microvilli on the cell surface (Figs. 2, 3). Especially transformed cells were smooth, without surface extensions. Normal cells were considerably larger than transformed cells and grew with widely extended leading lamellae. In contrast to normal cells, transformed cells – especially at confluent and superconfluent densities – had a compact appearance with very short leading lamellae. These data are in general agreement with those of Porter, Todaro & Fonte (1973) on attached 3T3 and SV3T3 cells.

Since in agglutination assays suspended cells are used, we also investigated the morphology of the cells in suspended form to determine whether the observed difference in size between normal and transformed cells *in situ* is reflected in the morphological appearance of the suspended cells and is relevant for the agglutination process. Therefore cells were suspended with EDTA as in agglutination assays. All suspended normal 3T3 cells had a surface adorned with many microvilli of varying length due to the EDTA detachment (Figs. 4, 6). Sometimes remnants of withdrawn leading lamellae and zeiotic blebs were also present. In contrast, most suspended SV3T3 cells were smaller, without microvilli but with some creases or folds in the cell membrane (Figs. 5, 7). Some transformed cells with a varying number of microvilli were also observed and occasionally zeiotic blebs were present.

Thus the difference in size between normal and transformed 3T3 cells *in situ* (Figs. 2, 3) seems to be reflected not only in the difference in diameter of the suspended cells but also in the number of microvilli present on the cell surface (Figs. 4–7). A detailed account of the investigation of the surface morphology of normal and

transformed fibroblasts during the cell cycle and during detachment with EDTA will be published elsewhere.

Cross-sections of suspended SV_3T_3 cells studied in the transmission electron microscope, generally showed a cell surface practically without extensions (Figs. 9, 11). Cross-sections of suspended normal $3T_3$ cells showed microvilli as extensions of the cell membrane or free from the adjacent outer membrane (Figs. 8, 10). These observations are in agreement with the surface morphology of the cells as seen in the scanning electron microscope (Figs. 4–7).

Estimation of the cell surface area of suspended normal and transformed fibroblasts

The scanning electron micrographs showed a clear difference in surface morphology between suspended normal and transformed cells, probably as a result of the large difference in size between these cells *in situ*. Making use of the transmission electron micrographs, we tried to estimate the effect of the surface extensions on the actual cell surface area of these cells.

Table 1. *Estimation of the cell surface area of suspended normal and transformed fibroblasts without (A) and with (B) microvilli*

	$3T_3$	SV_3T_3
A.		
Average radius of the cells (R, μm)	11	7
Cell surface area I (A-I) ($4\pi R^2$, μm^2)	1520	615
B.		
Average radius of the microvilli (r, μm)	0·07	0·07
Average length of the microvilli (h, μm)	0·92	0·62
Average density of the microvilli per μm^2 (d)	5·93	1·22
Total average surface area of the microvilli per cell (M) ($2\pi rh \times d \times A$-I, μm^2)	3645	205
Cell surface area II (A-II) (A-II = A-I + M, μm^2)	5165	820

Cell surface area I, based on the assumption that the suspended cells are smooth spheres, was calculated from measured diameters of cells as described in Materials and methods, and shown in Table 1 *A*.

Next, the increase in cell surface due to the presence of microvilli was estimated on cross-sections of the suspended cells (Table 1 *B*). The average length and thickness was measured on clearly cross-sectioned microvilli. The density of the microvilli was determined by measuring the circumference of the cell with a curvimeter. The average thickness of the sections was considered to be 0·1 μm. By counting the number of microvilli that were clearly continuous with the cell membrane, the number of microvilli per μm^2 surface area was obtained. With these data the total surface area of all microvilli present on the cell was calculated. Cell surface area II was determined by adding the surface area of the microvilli to cell surface area I (Table 1 *B*).

Binding of ^{3}H-concanavalin A on normal and transformed suspended cells

Normal and transformed 3T3 cells were suspended with EDTA and used in binding experiments as described in Materials and methods. The data are summarized in Table 2.

Table 2. *Binding of ^{3}H-Con A after 20 min at room temperature*

Cells	Conc. Con A, μg/ml	Molecules bound per cell, $\times 10^{-7}$	Molecules bound per μm^2 surface area I*, $\times 10^{-3}$	Molecules bound per μm^2 surface area II*, $\times 10^{-3}$
3T3	5	1·2	8	2·3
SV3T3	5	1·3	21	15·7
3T3	10	1·5	10	2 9
SV3T3	10	2·1	34	25·6
3T3	20	2·3	15	4·5
SV3T3	20	2 6	42	31 7
3T3	30	2·8	18	5 4
SV3T3	30	2·9	47	35 4
3T3	50	3·2	21	6·2
SV3T3	50	3·2	52	39·0

* Cell surface area I and II see Table 1.

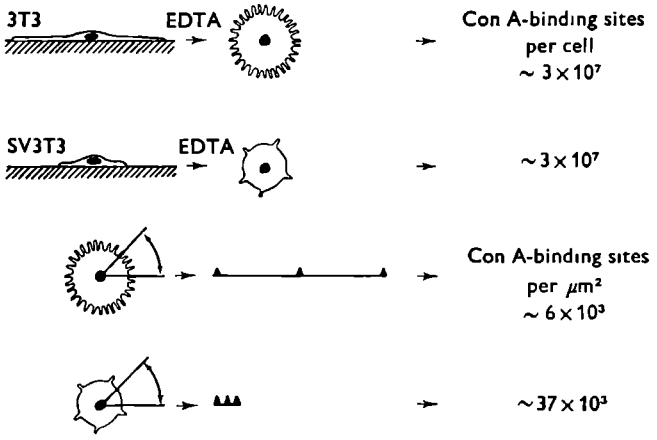

Fig. 1. Schematic representation of the effect of microvilli on the density of Con A-binding sites on 3T3 and SV3T3 cells.

In control experiments with α-MG no binding of ^{3}H-Con A occurred. Addition of α-MG 20 min after incubation with ^{3}H-Con A at room temperature maximally removed 60–80 % of the cell-bound Con A. This is in agreement with observations of Noonan & Burger (1973). The irremovable part of the cell-bound Con A has been ascribed to endocytosis. Little increase in ^{3}H-Con A binding was observed at concentrations higher than approximately 30 μg Con A per ml. Apparently, most of the Con A-binding sites had already been occupied at the lower concentrations. The small

increase at higher concentrations of Con A is probably due to endocytosis and perhaps also to binding on secondary binding sites with a lower affinity for Con A

In agreement with observations of others, we found that normal and transformed cells bind about the same number of Con A molecules per cell (Table 2). When the number of Con A molecules was calculated per μm^2 surface area I, SV3T3 cells had 2–3 times more cell-bound ^{3}H-Con A than 3T3 cells However, calculation of the number of cell-bound Con A molecules per unit of surface area II demonstrated an approximately 7 times higher density of binding sites on transformed than on normal cells

Fig 1 schematically summarizes the effect of the difference in size between normal and transformed 3T3 cells *in situ* and the resulting difference in surface morphology between suspended 3T3 and SV3T3 cells and indicates the consequences for the density of Con A-binding sites on these cells

DISCUSSION

The data presented here show a great size difference between normal and transformed 3T3 fibroblasts Similar observations have been made in the light microscope by other investigators in this field and have been described more specifically by Ben-Bassat *et al* (1971) in relation to agglutinability of cells Differences in extension of the leading lamellae have been demonstrated by us before on replicas of these cells (Collard *et al* 1975) When cells were suspended with EDTA as in agglutination assays, 3T3 cells were adorned with many microvilli, whereas most SV3T3 looked very smooth Because the spherical shape is the configuration with the smallest surface-to-volume ratio, the excess membrane material, resulting from the rounding-up of suspended cells, probably has been stored as surface extensions It has been suggested before that microvilli store surplus membrane material of cells (Follett & Goldman, 1970) This effect was particularly clear on 3T3 cells, growing with widely extended leading lamellae

The possible involvement of the surface morphology in the agglutination process has been suggested previously (Collard *et al* 1975) The data in this paper more specifically emphasize the contribution of differences in surface morphology to the actual cell surface area and thus to the density of the Con A-binding sites In binding experiments similar numbers of Con A-binding sites per cell were found on both cell types, in agreement with observations of others (Cline & Livingston, 1971, Ozanne & Sambrook, 1971, Arndt-Jovin & Berg, 1971, Ben-Bassat *et al* 1971, Inbar *et al* 1971) The cell surface area has generally been calculated on the assumption that suspended cells are smooth spheres, by measuring diameters in the light microscope (Noonan & Burger, 1973) or by determination of the wet volume of cells (Inbar & Sachs, 1969, Ben-Bassat *et al* 1971, Inbar *et al* 1971) Using these methods, the cell surface areas (cell surface area I) calculated by us agree with those of these authors the diameter of 3T3 cells is greater than that of SV3T3 cells, resulting in a 2–3 times larger cell surface area The density of Con A-binding sites, as expressed per cell surface area I, is therefore 2–3 times greater on transformed than on normal cells

However, the assumption that suspended cells are smooth spheres is not correct because at least part of the surplus membrane material after rounding up is not assimilated by an increased radius, but by formation of microvilli. Table 1 summarizes the differences in cell surface area and Table 2 the resulting differences in density of Con A-binding sites: transformed cells bind about 7 times more Con A molecules per unit surface area II than the untransformed parent cells.

The electron micrographs show that the total contribution of the microvilli to the surface area of detached 3T3 cells obviously is considerable. However, this contribution is difficult to quantitate exactly. In our approximation the effect of section thickness on counted microvilli per section and on measured length of microvilli has deliberately been left out of account. Section thickness may cause a certain overestimate of the number of microvilli, but this has been minimized by counting only clear cross-sections. With a section thickness of 0·1 μm (or thinner) and average diameter of the microvilli of 0·14 μm, the overestimate of the number of microvilli cannot be very great. On the other hand, curved microvilli will generally not be completely cross-sectioned by one section, resulting in a considerable underestimate of the length of the microvilli.

Although more Con A-binding sites were detected cytochemically in SV3T3 cells than in 3T3 cells, a 7 times higher concentration has not previously been found (Nicolson, 1971; Smith & Revel, 1972; Temmink, Collard, Spits & Roos, 1975). This discrepancy is probably due to a number of disadvantages of cytochemical methods like lack of quantitative detection (horseradish peroxidase) (Collard & Temmink, 1974) and all kinds of possible steric interferences (ferritin, haemocyanin) (Temmink *et al.* 1975).

It is as yet not known exactly how the agglutination response occurs. First it was supposed that transformed cells have exposed binding sites, whereas normal cells posses cryptic sites that can be unmasked by brief proteolytic treatment (Burger, 1969). Later the increased agglutinability of transformed cells was explained by Nicolson's observation that increased agglutination correlated with a clusterlike distribution of Con A sites on the cell surface. This irregular distribution was proved to result from rearrangement of these sites induced by addition of Con A and could be prevented by prefixation (Nicolson, 1971, 1973; Rosenblith, Ukena, Yin, Berlin & Karnovsky, 1973; Temmink *et al.* 1975). Because of the equal number of Con A-binding sites per cell and the observed differences in clustering of these sites on normal and transformed plasma membranes, it was postulated that the degree of clustering and thus the agglutination of cells was determined by the mobility of Con A-binding sites as resulting from the fluidity of the membrane (Nicholson, 1972, 1973; Rosenblith *et al.* 1973; Barnett, Furcht & Scott, 1974). However, this conclusion has been challenged by others (Smith & Revel, 1972; de Petris, Raff & Mallucci, 1973; Horwitz, Hatten & Burger, 1974; Temmink *et al.* 1975), and recently it has been found that clustering of Con A-binding sites may even, under certain conditions, hamper agglutination of cells (Rutishauser & Sachs, 1974).

Aside from possible steric interferences of microvilli directly with the agglutination process, our data indicate that differences in agglutination response between normal

and transformed cells may arise mainly from a difference in density of Con A-binding sites as originally suggested by Burger (1969). Moreover the density of the Con A-binding sites may also determine the degree of clustering and the size of the clusters formed. Thus our data seem to reconcile the old notion of increased density of Con A-binding sites with the more recent suggestion of cluster formation as a possible prerequisite for successful agglutination on transformed 3T3 fibroblasts.

Presently we are investigating the effect of trypsin on the surface morphology and thus on the density of Con A-binding sites in normal 3T3 cells as related to changes in agglutinability and changes in mobility of binding sites that trypsin is likely to have also.

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REFERENCES

ANDERSON, T. F. (1951). Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Trans. N.Y. Acad. Sci.* **13**, 130–134.

ARNDT-JOVIN, D. J. & BERG, P. (1971). Quantitative binding of ^{125}I-Concanavalin A to normal and transformed cells. *J. Virol.* **8**, 716–721.

BARNETT, R. E., FURCHT, L. T. & SCOTT, R. E. (1974). Differences in membrane fluidity and structure in contact-inhibited and transformed cells. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1992–1994.

BEN-BASSAT, H., INBAR, M. & SACHS, L. (1971). Changes in the structural organization of the surface membrane in malignant cell transformation. *J. Membrane Biol.* **6**, 183–194.

BURGER, M. M. (1969). A difference in the architecture of the surface membrane of normal and virally transformed cells. *Proc. natn. Acad. Sci. U.S.A.* **62**, 994–1001.

CLINE, M. J. & LIVINGSTON, D. C. (1971). Binding of ^{3}H-Concanavalin A by normal and transformed cells. *Nature, New Biol.* **232**, 155–156.

COLLARD, J. G. & TEMMINK, J. H. M. (1974). Binding and cytochemical detection of cell-bound Concanavalin A. *Expl Cell Res.* **86**, 81–86.

COLLARD, J. G., TEMMINK, J. H. M. & SMETS, L. A. (1975). Cell cycle dependent agglutinability, distribution of Concanavalin A binding sites and surface morphology of normal and transformed fibroblasts. In *Concanavalin A*, Proc. int. Symp. Norman, Oklahoma (ed. T. K. Chowdhury & A. K. Weiss), pp. 221–244. New York: Plenum Publications.

DE PETRIS, S., RAFF, M. C. & MALLUCCI, L. (1973). Ligand-induced redistribution of Concanavalin A receptors on normal, trypsinized and transformed fibroblasts. *Nature, New Biol.* **244**, 275–278.

FOLLETT, E. A. C. & GOLDMAN, R. D. (1970). The occurrence of microvilli during spreading and growth of BHK21/C13 fibroblasts. *Expl Cell Res.* **59**, 124–136.

HORWITZ, A., HATTEN, M. E. & BURGER, M. M. (1974). Membrane fatty acid replacements and their effect on growth and lectin induced agglutinability. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3115–3119.

HUET, Ch., LONCHAMPT, M., HUET, M. & BERNADAC, A. (1974). Temperature effect on the Concanavalin A molecule and on Concanavalin A binding. *Biochim. biophys. Acta* **365**, 28–39.

INBAR, M. & SACHS, L. (1969). Structural difference in sites on the surface membrane of normal and transformed cells. *Nature, Lond.* **223**, 710–712.

INBAR, M., BEN-BASSAT, H. & SACHS, L. (1971). A specific metabolic activity on the surface membrane in malignant cell transformation. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2748–2751.

NICOLSON, G. L. (1971). Difference in topology of normal and tumour cell membranes shown by different surface distributions of ferritin-conjugated Concanavalin A. *Nature, New Biol.* **233**, 244–246.

NICOLSON, G. L. (1972). Topography of membrane Concanavalin A sites modified by proteolysis. *Nature, New Biol.* **239**, 193–197.

NICOLSON, G. L. (1973). Temperature-dependent mobility of Concanavalin A sites on tumour cell surfaces. *Nature, New Biol.* **243**, 218–220.

NICOLSON, G L. (1974). The interactions of lectins with animal cell surfaces. *Int. Rev. Cytol.* **39**, 89–190.

NOONAN, K. D. & BURGER, M. M. (1973). Binding of ^{3}H-Concanavalin A to normal and transformed cells. *J. biol. Chem.* **248**, 4286–4292.

OZANNE, B & SAMBROOK, J. (1971). Binding of radioactively labelled Concanavalin A and wheat germ agglutinin to normal and virus-ransformed tcells. *Nature, New Biol.* **232**, 156–160.

PORTER, K. R., TODARO, G. J. & FONTE, V. (1973). A scanning electron microscope study of surface features of viral and spontaneous transformants of mouse Balb/3T3 cells *J. Cell Biol.* **59**, 633–642.

ROSENBLITH, J. Z., UKENA, T. E., YIN, H. H., BERLIN, R. D. & KARNOVSKY, M. J. (1973) A comparative evaluation of the distribution of Concanavalin A binding sites on the surfaces of normal, virally-transformed, and protease-treated fibroblasts. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1625–1629.

RUTISHAUSER, U. & SACHS, L. (1974). Receptor mobility and the mechanism of cell-cell binding induced by Concanavalin A. *Proc natn. Acad. Sci. U.S.A.* **71**, 2456–2460.

SMETS, L. A. & DE LEY, L. (1974). Cell cycle dependent modulation of the surface membrane of normal and SV-40 transformed 3T3 cells. *J. cell. Physiol.* **84**, 343–348.

SMITH, S. B. & REVEL, J-P. (1972). Mapping of Concanavalin A binding sites on the surface of several cell types. *Devl Biol.* **27**, 434–441.

TEMMINK, J. H. M., COLLARD, J. G., SPITS, H. & ROOS, E. (1975). A comparative study of four cytochemical detection methods of Concanavalin A binding sites on the cell membrane. *Expl Cell Res.* (in Press).

(*Received* 26 *March* 1975)

Fig. 2. Scanning electron micrograph of attached confluent 3T3 cells; × 1000.

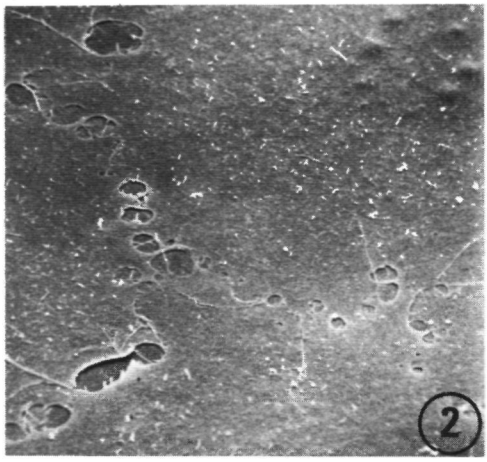
Fig. 3. Scanning electron micrograph of attached confluent SV3T3 cells; × 1000.

Fig 4. Scanning electron micrograph of 3T3 cells suspended with 10^{-5} M EDTA; × 1000.

Fig 5. Scanning electron micrograph of SV3T3 cells suspended with 10^{-5} M EDTA; × 1000.

Fig. 6. Like Fig. 4; × 2500.

Fig. 7. Like Fig. 5; × 2500.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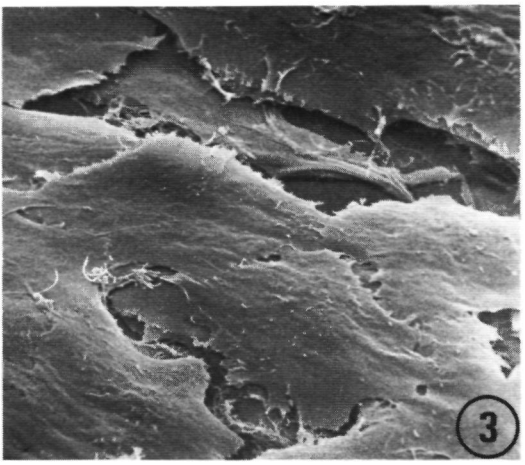
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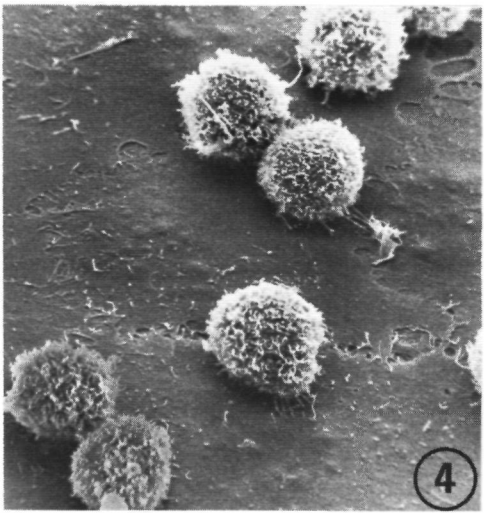
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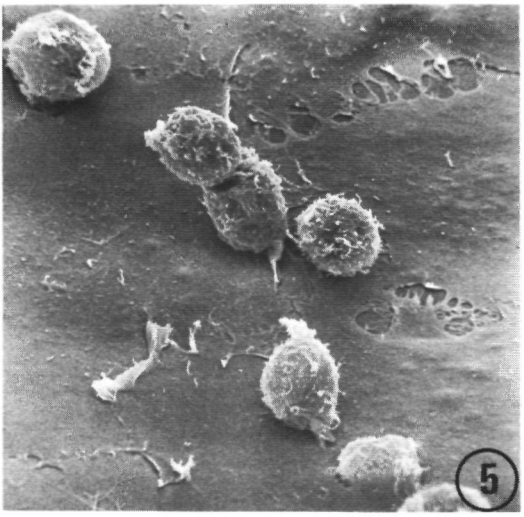
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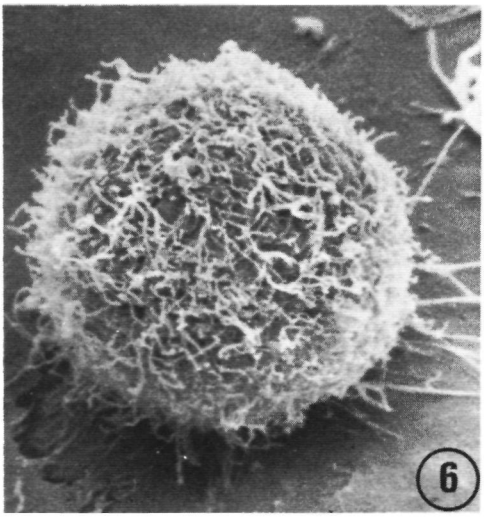
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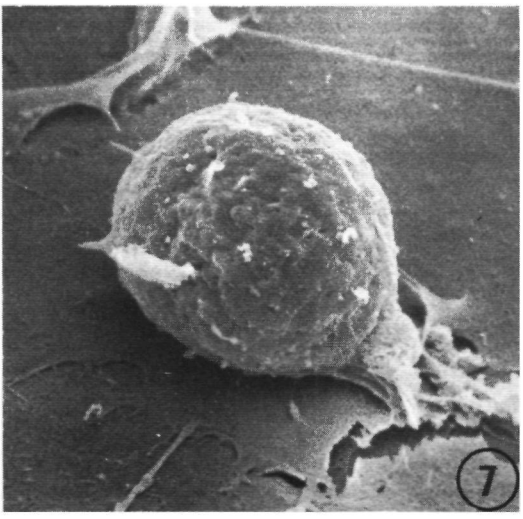
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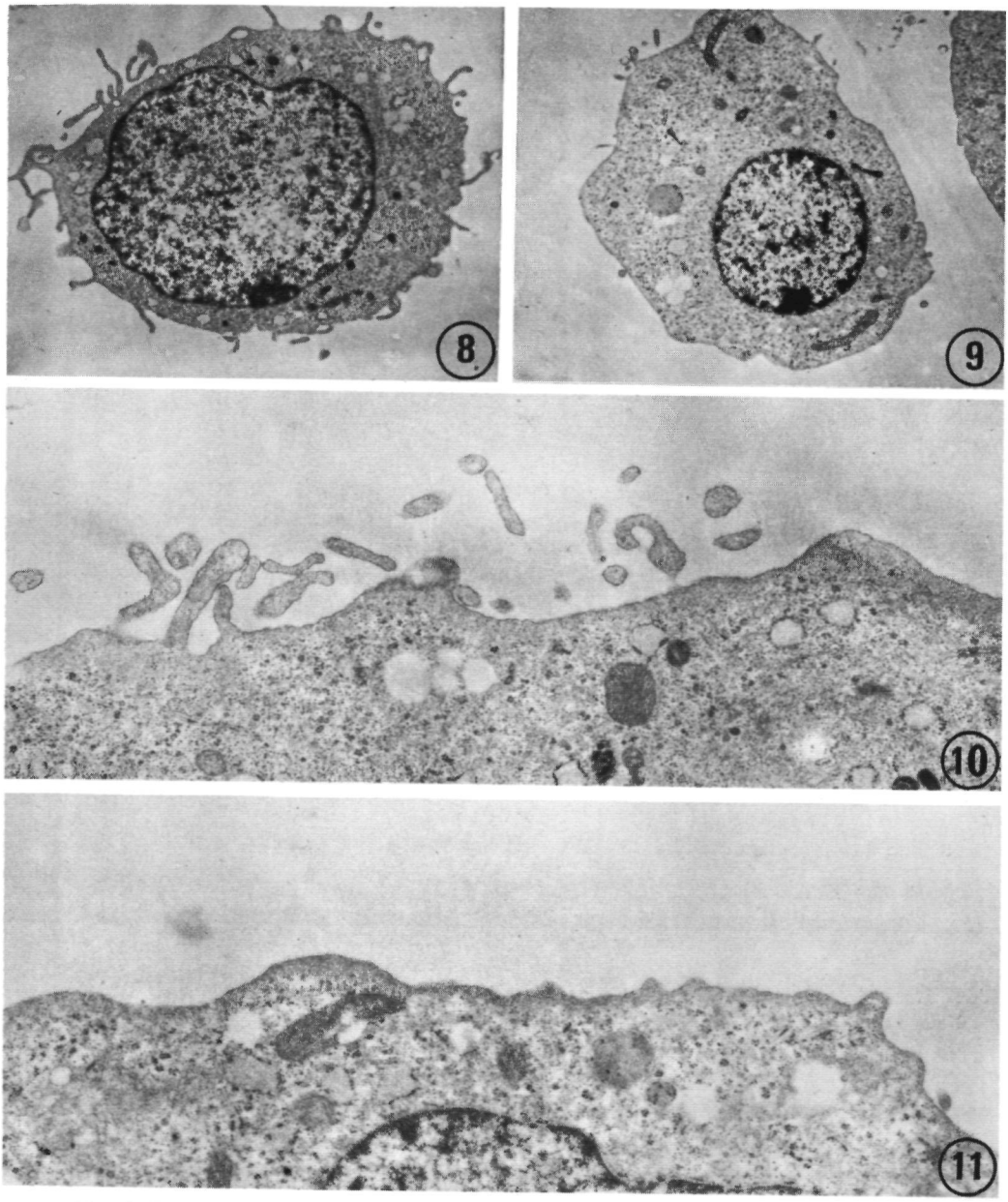

Fig. 8. Transmission electron micrograph of cross-sectioned $3T3$ cell; ×4800.

Fig. 9. Transmission electron micrograph of cross-sectioned $SV3T3$ cell; ×4800.

Fig. 10. Transmission electron micrograph of part of the cell membrane of a $3T3$ cell; ×14500.

Fig. 11. Transmission electron micrograph of part of the cell membrane of a $SV3T3$ cell; ×14500.

STELLINGEN

Conclusies over phenotypische reversie van getransformeerde cellen naar een normaal groeigedrag *in vitro*, dienen niet alleen gebaseerd te worden op morphologische kenmerken

Dit proefschrift Publicaties I en II

II

Verschil in redistributie van Concanavaline A bindingsplaatsen op de plasmamembraan van normale en getransformeerde fibroblasten kan niet als hoofdoorzaak beschouwd worden van het verschil in agglutinatiegedrag tussen deze cellen

Dit proefschrift Publicatie V

III

De conclusie van Willingham en Pastan dat de aanwezigheid van microvilli op het celoppervlak van getransformeerde fibroblasten de verhoogde lectine geïnduceerde agglutineerbaarheid veroorzaakt is in haar algemeenheid aanvechtbaar

Willingham, M C & Pastan, I (1975) *Proc Natl Acad Sci USA* 72 1263-1267

IV

Uit de resultaten van v Beek *et al* kan worden afgeleid dat de extra sialylering van bepaalde glycoproteinen aan het oppervlak van tumorcellen mogelijk een waardevol criterium kan zijn ter bepaling van het al of niet maligne karakter van transformatie *in vitro*

v Beek, W P , Smets, L A & Emmelot, P (1973) *Cancer Res* 33 2913-2922
v Beek, W P , Smets, L A & Emmelot, P (1975) *Nature* 253 457-460

V

Bij het zoeken naar tumorvirus in menselijk tumormateriaal in weefselkweek dient rekening gehouden te worden met de mogelijkheid dat virus of virusachtige deeltjes reeds in het serum van het gebruikte weefselkweekmedium aanwezig zijn

Benz, E W & Moses, H L (1974) *J Natl Cancer Inst* 52 1931-1933
Fong, C K Y , Gross, P A , Hsiung, G D & Swack, N S (1975) *J Clin Microbiol* 1 219 224

VI

De conclusie van Jutsum *et al* dat de toename van de lipidconcentratie in de haemolymphe van de treksprinkhaan *Locusta migratoria*, tijdens hongering niet veroorzaakt wordt door afgifte van het adipokinetisch hormoon uit de corpora cardiaca, wordt door hun experimenten niet afdoende bewezen

Jutsum, A R , Agarwal, H C & Goldworthy, G J (1975) *Acrida* 4 47 56

VII

De hypothese over de relatie tussen microfilamenten en microtubuli enerzijds en de beweeglijkheid van plasmamembraan componenten anderzijds, zal aan betekenis winnen wanneer directe verbindingen tussen beide systemen submicroscopisch kunnen worden aangetoond

Yahara, I & Edelman, G M (1975) *Proc Natl Acad Sci USA* 72 1579 1583

VIII

Gelden verworven middels professioneel gevoerde acties, zoals de actie "Geven voor Leven", dienen even professioneel beheerd en toegekend te worden.

IX

Interlandelijke adoptie, mits op verantwoorde wijze uitgevoerd, is een oplossing voor acute sociale en materiele nood van wezen en verlaten kinderen uit de derde wereld; het draagt echter aan de structurele wijziging van de maatschappelijke oorzaken van die nood even weinig bij als het zenden van voedsel bijdraagt aan het wegnemen van de oorzaken van hongersnood.

X

Aan het besluit tot sluiting van het Herseninstituut is wellicht te weinig hersenwerk voorafgegaan.

J.G. Collard

Nijmegen, 20 februari 1976